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# The Incidence of Primary Carcinoma of the Liver in the Southern Bantu. I. Critical Review of the Literature [1]

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A high relative frequency of carcinoma of the liver among different sections of the Negro (Bantu) population of Southern Africa has often been reported and, in view of the relative rarity of this disease among European races, is recognized as a challenging problem in the demography of cancer.

Unfortunately, certain limitations of the published statistics seem to have been disregarded, and a rather distorted view of the real incidence is prevalent. A recent leading article in *The Lancet* (*1*), for example, stated that "In South African natives primary cancer of the liver constitutes over 50 percent of all forms of cancer." Presumably this was a slight misquotation of Berman (*2*), whose figures actually referred to percentage of carcinomas, not of all cancers. Nevertheless this report is understandable, as the figure of 50 percent had been published in South Africa, is still generally accepted (*3*), and has not been refuted.

The limitations of the published South African figures are well recognized among pathologists in South Africa, as well as by critical observers outside the country. Steiner (*4*), for example, states, "It is important to remember that nearly all reported data deal with frequency relative to other tumors and not with true incidence rate or prevalence in standardized populations." Dorn (*5*) has pointed out that existing data are "highly selective and do not provide a valid basis for generalizing to the total population," concluding tentatively that "the incidence of primary liver cancer probably is somewhat higher among Negroes in Africa than it is among Negroes in the United States. However, the incidence rate is comparatively low in both populations." Gilliam (*6*) has also drawn attention to the fallacies in some of the South African literature, adding "Only those in position to make local and critical appraisal of these factors can estimate the sturdiness of these reeds upon which reliance must be placed." This the present paper attempts to do.

It should not be necessary to explain that critical examination of earlier work is not intended to detract from the credit due to those whose energy

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and imagination led them to gather and publish facts when the potentialities for cancer research in South Africa were little realized. The pitfalls of cancer statistics were not so obvious then, or, if recognized, fortunately did not dissuade the authors from drawing attention to clinical impression. Des Ligneris (*7*), for example, freely admitted that "The time has not yet come to give a complete and true picture of cancer in South African natives."

Preliminary studies, however, have served their purpose in drawing attention to a condition and its peculiar features. They should, thereafter, be replaced by more critically designed investigations. Most of the earlier references to liver cancer in South Africa are listed in Berman's publications (*2,8,9*), but he did not comment on the relative value of these studies, their probable bias, nor the stringency of the criteria employed in diagnosis when he derived the hepatoma/carcinoma [3] ratio of 50 percent. This was a weighted average: Its bias will become more apparent below, but briefly it derived from the fact that the greatest proportion of his population consisted of young males from the Union and Mozambique, while the remainder were drawn from various general populations of the Union of South Africa, Portuguese East Africa, Kenya, Tanganyika, Uganda, Belgian Congo, Fernando Po, and French Equatorial Africa.

Many sources of bias may be encountered in studies of diseases of the Bantu (*10*). If data are not statistically comparable the exact percentage from the addition of haphazard clusters cannot be taken too seriously, though they may demonstrate without reasonable doubt that hepatoma is common among these people. Epidemiologic statistics, however, should permit exact comparisons between different groups. This requires, first, that accuracy of the diagnostic methods employed shall be known, or at least comparable, and, second, that the characteristics of the parent populations from which the samples are drawn shall be defined, at least as regards age and sex composition. In both these respects the literature on malignant hepatoma in the Bantu is deficient. The figures permit the calculation of ratios only (hepatomas per carcinoma, malignant tumor, necropsy, or hospital admission) in different groups. Age-specific rates were calculated by Berman for the age group 18 to 45 [(*2*) pp. 7, 9] but, as he included some cases over 50 years (*11*), even these rates are not very precise—quite apart from the wide range of the age group, which is excessive in view of uneven distribution of ages within the group.

It is desirable, therefore, that this literature be re-examined in detail, with critical comments, especially as the original articles are not easily obtainable outside of South Africa. Additional evidence from Lourenço Marques will also be included. In a subsequent paper the preliminary results of an attempt to obtain more satisfactory statistics for the Johannesburg Bantu population will be presented.

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[3] The term "hepatoma" is used in this paper as synonymous with "primary carcinoma of the liver" and includes both hepatocellular and cholangiocellular types.

## Critical Review of Information Relating to Hepatoma in the Southern Bantu

Cancer studies in primitive races tend to pass through similar developmental stages, and the growth of our knowledge of hepatoma in the Bantu is no exception to the general pattern. First there are explorers who, after a relatively short stay with a primitive race, conclude that cancer does not occur. They are followed by missionary doctors and district surgeons. Resistance and superstition tends to keep older age groups away from these doctors who, consequently, very seldom encounter cancer. Apart from isolated case reports, their statements take the form "After so many years of practice in this district I saw only so many cancers. By comparison with its occurrence in European populations, cancer appears to be very rare in the natives." As mission and other hospitals are built, cancers are encountered more often, although still infrequently; their figures are presented as ratios of cancers to other cases, *e.g.*, to hospital admissions. Interpretation of such figures is difficult, as the pattern of disease differs greatly from that of more civilized regions, preventable diseases are much commoner, and social attitudes and treatment facilities affect hospital admissions. As laboratory and other services improve, other studies of cancer ratios follow, *e.g.*, sex ratios (these at first ignore the sex ratios in the parent populations), necropsy, and biopsy ratios, all of which are compared directly with those obtaining in civilized communities—and inevitably are found to be different. Indeed it would have been more surprising to find the cancer ratios similar, since in the first place the age distribution of the primitive population invariably differs considerably from that of civilized communities. At length the attempt is made to relate the results to the parent population, culminating in age- and sex-specific rates and ratios, that for the first time enable direct demographic comparisons to be made, and lead eventually to the final stage of comparing rates in environmental or cultural groups within the primitive people.

For a disease as common as hepatoma is generally believed to be, the early literature on this neoplasm in the Bantu is remarkably deficient (*12,13*). Livingstone (*14*) noted that cancer was quite unknown among the Bakwains (Kwena tribe) of Bechuanaland (p. 127) and also in the Makoloto in Northern Rhodesia, north of the Chobe and Zambesi Rivers (p. 505). I can, in fact, find no 19th-century references to hepatoma in the Bantu.

The 1901 (*15*) report of the medical superintendent of Addington Hospital, Durban, records 5 cancers of the liver in 16 malignant diseases treated, but does not state what race was affected. In the report for 1904 (*16*), native patients were distinguished; 1 carcinoma of the breast and a sarcoma were recorded among 694 native in-patients but no hepatomas.

The first authenticated report of hepatoma in indigenous races came from Grahamstown, in the Eastern Cape Province, in 1905, when Bruce-Bays (*17*) described the fourth case of hepatoma under his care, in a "Kaffir," *i.e.*, one of the Bantu, about 40 years of age, who died suddenly from intra-



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peritoneal hemorrhage. The case was confirmed by necropsy, the liver weighing 7 lbs. 7 oz. (3,373 gm.), half consisting of neoplasm. Microscopically the tumor was diagnosed as multiple cylindrical carcinoma. The detailed description suggests either an adenoid hepatocellular or cholangiocellular hepatoma.

In 1906 (*18*) Hawes described 3 cases in Hottentots from the same town, all being "of the better type." (He does not say whether this refers to their social standing, nutrition, or dilution with white ancestry, but possibly includes all three.) All cases were confirmed by necropsy and histologic examination, and the published photomicrographs enable more up-to-date histopathologic diagnosis. The first, a male 17 years old, had what appears to have been a cholangiocellular tumor, associated with biliary cirrhosis, and bile-duct proliferation. The second was a female, age 32, with hepatocellular tumor and cirrhosis. The third, a male, age 16, revealed a hepatocellular hepatoma, partly adenoid, associated with coarse cirrhosis without bile-duct proliferation. These tumors in all cases were nodular and appeared to start in the right lobe. The clinical course was short. Hawes emphasized the early ages of incidence and the striking fact of so rare a condition appearing in a race in which tumors of any kind were regarded as uncommon. These detailed studies were made possible by the presence and assistance of the Colonial Bacteriological Institute in Grahamstown.

*Comment.*—It is noteworthy to what extent studies on hepatoma have been dependent on the availability of a pathologic institution. The Colonial Bacteriological Laboratory in Grahamstown was set up in 1892 for study of animal diseases, but occasionally tumors were investigated for private medical practitioners [Edington, report (*19*)]. Thus the first well documented South African records of hepatoma were made possible. Since the closure of the Institute, to my knowledge, not a single case of hepatoma has been reported from Grahamstown.

The cases reported by Hawes were all in Hottentots, a race distinct from the Bantu, and, at this time and place, certainly racially admixed with Europeans. In South Africa, however, the term "Hottentot" is often loosely applied to lighter-skinned persons and might include Cape Malays or Bantu-European mulattoes, as well as persons derived from Hottentot or Bush ancestors. The association of the tumor with cirrhosis in all cases is striking.

The first attempt to survey the incidence of malignant disease in the Bantu was made by the Natal Cancer Research Committee, formed in October 1905 (*20*), which submitted reports in 1907 (*21*) and 1910 (*22*). They attempted to record all cases of malignant disease in European, Indian, and Bantu populations of Natal and Zululand. In this, as they acknowledge, they failed. The Bantu population was estimated for 1908 as 976,222, of which only 13.7 percent were 40 years of age and over. In the 4-year period 1906–09, 5 deaths from cancer were recorded in pure Bantu, 3 diagnoses being confirmed microscopically. [I have excluded 3 cases, *viz.*, a Griqua (one of the Hottentot tribes), a St. Helena Negress, and a half-caste, since all these would certainly have been of mixed Euraf-rican descent.] Including these mixed cases the cancer death rate was calculated as 0.21 per 100,000 per annum (*20*); excluding those of mixed

race, it is approximately 0.13 per 100,000. Two of the 5 Bantu cases suffered from hepatomas, confirmed microscopically. In addition, 20 cancers were diagnosed by biopsy—none were hepatomas. Clinical records of another 8 melanotic sarcomas of the foot, diagnosed clinically in 1905 and 1906, were also mentioned by Watkins-Pitchford (*22*).

*Comment.*—The quantitative results may be ignored, yet it is interesting that in this first survey they encountered many cancers which subsequent demographic studies suggest may be significantly frequent in the Bantu races (carcinomas of liver, cervix uteri, and conjunctiva; rodent ulcer in an albino Bantu; and melanotic sarcomas of the foot).

In 1910, Dr. G. A. Casalis reported to the South African Medical Congress that he attended an operation by Dr. Temple Mursell in Johannesburg on a pure Mozambique boy for adenocarcinoma of the liver (*23*).

*Comment.*—This is the first reference to hepatoma in the Mozambique Bantu, a group later shown to be particularly susceptible.

It is interesting to note that 2 reports of cancer in the Bantu of Basutoland (*23*) and the adjoining Orange Free State (*24*) do not mention hepatoma among these people. They do not state whether necropsies were carried out, so it is possible that their cases were limited to cancers of the accessible organs.

A series of reports of the Johannesburg Medical Officer of Health, Charles Porter, recorded high frequencies of cancer of the liver since 1910 (report for 1 July 1906–30 June 1909 [(*25*), pp. 22, 23], when cancer of the liver was mentioned as the cause of death in 21 out of 48 deaths from cancer in South African Colored. In the term "Colored," Porter embraced Bantu (the preponderant group) and the mixed races, but excluded Asiatics. In his report for the year ending 30 June 1911 (*26*), the Bantu and mixed populations were distinguished. In the Bantu, hepatoma was recorded as the cause of death in 17 out of 26 due to cancer. Each subsequent report mentioned numerous liver cancers in the Bantu and also in Eurafricans (*27–29*). From the 1917 (*30*) report (p. 10) and from an annotation in the *Transvaal Medical Journal* (*31*), it is evident that these figures included miners in the Johannesburg area. From 1921 onward, "non-resident Bantu" were distinguished and accounted for about a third of the deaths from cancer. Presumably these were miners, but the percentage of hepatomas occurring in nonresidents is unfortunately not stated.

In 1925 (*32*) this report analyzed the cancer mortality in Johannesburg for the decennium July 1914 to June 1924, and showed the percentages for whites to range from 0.62 per 1,000 to 0.78 per 1,000. The corresponding figure for Bantu was as low as 0.08, and Porter comments that the population was composed mainly of young males, the ratio of females to males being about 1:5.5. He also calculated the ratios of different cancers as causes of death, and, among the Bantu, cancer of the liver represented 52.04 percent, that of the stomach coming second with 24.56 percent.

*Comment.*—Despite the remarkable observations of mortality in these reports, Porter made no comment on the frequency of hepatoma, the crude

absolute incidence of which can be calculated from his crude cancer death rate (0.08/1,000) and the hepatoma ratio (52.04%) as 4.16 per 100,000 of this preponderantly young male population. These observations, referring as they do to a mixed urban and mining population of both sexes, are difficult to interpret and were more or less ignored, Berman being the only author to refer to them. The high proportion of cases of carcinoma of the stomach mentioned in this population is open to question in the light of subsequent information on these people.

Attention was first drawn to the relative frequency of hepatoma in the Bantu in the Annual Report for the South African Institute for Medical Research, for 1920 [(*33*), p. 34], presumably by Pirie, who was then Superintendent of the Routine Division of this Institution. Pirie (*34*) analyzed the histopathologic material submitted from Bantu patients between 1912 and 1920, finding 46 sarcomas and 93 carcinomas, 52 of which had been diagnosed as hepatoma. Although he rejected 16 hepatomas as unproved, this remained the commonest carcinoma. Of the 36 cases whose diagnosis he accepted, 28 were hepatocellular, 3 cholangiocellular, and 5 mixed (of these, 3 were probably hepatocellular and 2 probably a mixture of hepatocellular and cholangiocellular). As his youngest case was a Zulu of 10 years, he concluded that diet was probably not responsible for the tumors. He admitted that the material was not a fair cross section, since most came from young males (the Institute was then handling a large amount of material from the mines). However, he found for hepatomas "young adult males (mine boys) undoubtedly predominating." Of the 29 male cases, 20 are recorded as miners. In 14 the age is given, *viz.*, "about 10," 14, 19, 19, 20, 23, 25, 29, "about 30," 32, 37, and 3 "about 50," *i.e.*, cases occurred with approximately equal frequency in each decade from the second to the fifth. The state of the liver could be assessed in 33, and of these 31 were demonstrably cirrhotic. Since ova of schistosomes had been detected in 10 of the 36 cases, and in 4 were found in the liver substance, Pirie suggested that schistosomiasis might be a common cause of this cirrhosis, though not the only cause. In support of this, Pirie quoted Turner and Brebner's findings (*35*) that schistosomiasis was encountered in 56 out of 57 East Coast natives (Mozambique and Nyasa) dying of cerebrospinal meningitis, and in 8 of the 56 the liver was macroscopically cirrhotic. Pirie added that in 16 cases at this Institute schistosomiasis was associated with cirrhosis, though extreme pipe-stem cirrhosis was not encountered. He postulated that schistosomiasis was probably the most common cause of the cirrhosis of the liver so commonly seen in South African natives, and, as such, was a factor in the causation of the relatively common hepatic carcinoma occurring among them (p. 90). This view was modified, presumably by Pirie, in the Annual Report of this Institute for 1922 (*36*) where it is stated that 19 further cases of primary carcinoma of the liver had been noted, of which 6 showed ova and 6 did not give any indications of either past or present infestation. The writer therefore concluded that "although Schistosomiasis may be one factor in accounting

for the striking relative frequency of primary cancer of the liver amongst South African Natives, it is by no means the only one." (p. 34).

*Comment.*—Pirie's studies very strikingly demonstrated a high frequency of hepatoma in the Bantu. The ages quoted indicate that the tumors may develop at a remarkably early age for carcinoma, but also, in view of the young age distribution of the population, could suggest that the condition, like most other carcinomas, becomes commoner with increasing age. He noted its predominantly hepatocellular character—under 10 percent being purely cholangiocellular, and its frequent association with cirrhosis of the liver (94% of 33 cases). His conclusions on the probable association between schistosomiasis and cirrhosis may be questioned, for schistosomiasis was exceedingly common (98%) in East Coast natives. The demonstration of schistosomiasis in 16 out of 55 patients with hepatomas therefore may indicate no more than the presence of East Coast natives in this series.

Pirie's publication revived interest in malignant diseases among the Bantu, and several references to hepatoma in mine Bantu followed (*37-40*). In 1924 Leipoldt (*41*) wrote a note warning against "unwarranted speculation" in discussions on the etiology of cancer, commenting that the confession of certain medical men to having seen little or no cancer among such primitive peoples "has a certain negative value, as much, perhaps, as that of a witness for the defense who states that he never saw the prisoner commit the crime." From his own experience he quotes 2 sarcomas in 2,580 native patients and includes figures supplied to Dr. Brock by Dr. Macfarlane (*42*), Principal Medical Officer for Basutoland, giving the cancers seen in 1921 and 1922. Seventy-two cancers were reported, which included 5 sarcomas of the liver and 2 carcinomas of this organ. Malignant disease of the liver (7 cases) was exceeded in frequency by carcinoma of the rectum (19 cases) and of the uterus (8 cases).

*Comment.*—The method of diagnosis in Macfarlane's series is not given, nor are the hospital size and number of patients studied. The diagnoses of 5 sarcomas of the liver may be doubted without more evidence, as hepatic sarcoma is extremely rare; these tumors were probably carcinomas. The accuracy of the series is also open to doubt in view of the variation recorded between successive years—in 1921 no epitheliomas of the rectum were recorded whereas in 1922 there were 19.

In 1925 MacVicar (*43*) reported 141 cases of malignant disease in 10,000 in-patients treated at the Victoria Hospital, Lovedale, which serves a rural Bantu population, predominantly of the Xhosa tribes in the Eastern Cape Province. The site of origin was known in 127 cases, 97 being carcinomas, and, of these, hepatomas preponderated (22 cases). The method of diagnosis was not given. MacVicar commented that "cancer of the liver is common in comparatively young men, and even boys," the age distribution being 13 to 68 years, the average being 44 years, and the sex ratio 10:1 in favor of males. Schistosomiasis was regarded as the etiologic factor.

*Comment.*—Age and sex distribution of the hospital patients was not mentioned, nor the stringency of the diagnostic methods. The ratio of hepatoma to cases of malignant disease was 22:141 (15.6%) and hepatoma to carcinoma 22:97 or 22.7 percent. This tumor represented 0.22 percent of

admissions. It is noteworthy that from a relatively older population, the average age is greater, and the maximum age higher, than in Pirie's series.

In 1927 the first of des Ligneris' surveys of cancer in the Bantu patients at Elim Hospital, Northern Transvaal, was published (*44*). The report covered the period 1906–26 (13,170 in-patients) and the entire material was confirmed histologically, necropsy being performed in 90 percent of deaths. Of 97 malignant tumors, there were 58 sarcomas, 17 melanomas, 6 carcinomas of the skin, 15 mixed tumors of salivary glands, and 1 neuroglioma. In addition, there was an adenocarcinoma whose origin could not be ascertained beyond that it arose in liver, pancreas, or stomach. No definite case of hepatoma was reported, despite the marked prevalence of cirrhosis and schistosomiasis in the population.

*Comment.*—The absence of hepatomas from this series is striking. Attempts have been made to explain it on the grounds that the male population at Elim was poorly represented, that adult males attended witch doctors, and that mainly young women and children attended this hospital [Keen (*45*)]. This view is not based on any direct evidence nor is it supported by the sex ratio of 44 males to 53 females given in des Ligneris' paper.

Dr. Rosset, who is at present Superintendent of Elim Hospital, permitted me to examine the records, and the results of samples drawn from the hospital population are submitted in table 1. The ages stated in the hospital records represent rough estimates and cannot be regarded as precise.

It is evident, however, that if there has been a trend over the years, it has been toward greater attendance by younger patients, for whom a marked increase may be noted. Maternity services have also grown steadily, as evidenced by the number of newborns. In most years there was a preponder-

TABLE 1.—*Survey of Bantu patients at Elim Hospital, according to age and sex*

| | 1923–24 | | 1929–30 | | 1934–35 | | 1953 & 1954 | |
|---|---|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Newborn | 1 | 0 | 6 | 6 | 6 | 8 | 40 | 57 |
| 0–4 | 8 | 6 | 9 | 9 | 13 | 6 | 43 | 37 |
| 5–14 | 12 | 13 | 20 | 13 | 22 | 14 | 49 | 28 |
| 15–24 | 24 | 30 | 24 | 31 | 41 | 14 | 50 | 78 |
| 25–34 | 18 | 29 | 22 | 62 | 46 | 32 | 25 | 78 |
| 35–44 | 19 | 26 | 32 | 32 | 17 | 19 | 20 | 27 |
| 45–54 | 9 | 10 | 14 | 17 | 5 | 10 | 13 | 11 |
| 55–64 | 6 | 7 | 2 | 5 | 7 | 5 | 4 | 16 |
| 65–74 | 4 | 2 | 4 | 2 | 2 | 1 | 9 | 7 |
| 75–84 | 1 | 0 | 0 | 0 | 0 | 0 | 7 | 1 |
| Unknown | | | | | | 1 | | |
| Total in sample* | 102 | 123 | 133 | 177 | 159 | 110 | 260 | 340 |
| Total patients | 1, 132 | | 1, 558 | | 1, 349 | | 12, 000 | |
| Totals | | | | | | | | |
| 0–14† | 20 | 19 | 29 | 22 | 35 | 20 | 92 | 65 |
| Sex ratio | *1. 05* | | *1. 31* | | *1. 75* | | *1. 41* | |
| 15–34 | 42 | 59 | 46 | 93 | 87 | 46 | 75 | 156 |
| Sex ratio | *0. 71* | | *0. 49* | | *1. 89* | | *0. 48* | |
| 35+ | 39 | 45 | 52 | 56 | 31 | 36 | 53 | 62 |
| Sex ratio | *0. 87* | | *0. 93* | | *0. 86* | | *0. 85* | |

*Sample totals in each period represent 20% of hospital admissions except for 1953 and 1954 where they comprise only 5%.

†Excluding newborns.

ance of females between the ages of 15 and 34, which may be explained by the tendency for young males to be drawn away by employment in mines and industries elsewhere, but the number of older males attending the hospital is adequate confirmation of des Ligneris' contention that he did not think "that Natives who come to consult the white medicine-man for all sorts of internal troubles which were uninfluenced by Native medicines would fail to come just when the trouble is cancer (of which they know nothing), whilst they came in large numbers when their bad state of health was due, for example, to tuberculosis." (*44*).

How then can the apparent increase in liver cancer at Elim Hospital be explained? It seems not unlikely to the writer that much of this apparent increase can be attributed to secular changes in the spectrum of diseases treated in admissions to this hospital. In 1923–24, venereal diseases accounted for 70 percent of admissions, in 1929–30 the figure was 59 percent, in 1934–35 it was 52 percent, while in recent years the proportion has become insignificant, as a result of the introduction of penicillin therapy. The hospital populations for these different periods are not really comparable, therefore, and even in the absence of a real change in cancer incidence one might expect to find an apparent increase in cancer frequency.

In the same year Beyers (*46*) analyzed the admissions to the non-European wards of the Johannesburg Hospital between 1921 and 1926. Of 106 cases of malignant disease there were 75 carcinomas, 22 being hepatomas, and he concluded that hepatoma was the most common form of malignant disease among the Bantu, the average age being 40 and the youngest 17.

*Comment.*—The method of diagnosis is not stated, but Beyers says that he excluded all doubtful cases and enumerated "only those where the diagnosis rests on pathological or operation findings or on reasonable clinical grounds." He did not comment on the adequacy of hospital records. Although details are not given regarding the age and sex composition of the hospital population, it seems probable that there was a distinct preponderance of male patients at that time, although the figure of 5.5 males to every female in the city given by the Medical Officer of Health (*32*) included the Bantu miners in the city area, who would have been treated at Mine hospitals and were thus omitted from Beyers' study. Beyers' publication had the unusual distinction of being accompanied by an editorial disclaimer (*47*), which, severe though it may seem, indicates that the limitations of such statistics were well appreciated in 1927.

Beyers also quoted figures provided by Orenstein (*48*) derived from 13 mine hospitals on the Witwatersrand for the 6 years 1919–25, covering 200,000 patients. Among these, 49 cases of malignant disease were diagnosed, 37 being carcinomas and 12 sarcomas. Thirty carcinomas and 8 sarcomas were said to have arisen in the liver.

*Comment.*—Probably all these hepatic "sarcomas" were primary carcinomas of the liver; if so, hepatoma accounted for 38 out of 49 malignancies (78%) and 38 out of 45 carcinomas (84%). This was the first demonstration, apart from Pirie's evidence, that the ratio of hepatoma to other malignant tumors was extraordinarily high in Bantu miners.

In 1929 (*49*) and 1932 (*50*), Fischer published 2 series of consecutive necropsies carried out on Bantu miners at the City Deep Central Native Hospital, Johannesburg, over the 10-year period 1922–31 inclusive.

Accidental deaths are excluded. A total of 1,963 necropsies was carried out by 16 mine medical officers (apart from Fischer, not pathologists by training) and histologic examinations were made at the South African Institute for Medical Research. In the first report (*49*) on 1,042 cases, it is stated that multilobular cirrhosis was found in 10 percent of all East Coast natives and in 2.5 percent of all Union natives. The first series yielded 13 malignancies, 9 out of the 11 carcinomas being hepatomas, of which 7 were confirmed histologically. All these carcinomas occurred in Portuguese East African natives. In the second report (*50*), it was noted that schistosomiasis was found in consecutive necropsies on 66.5 percent of 185 East Coast natives and in 2.1 percent of 141 Union natives examined. Over the whole period they found 22 malignancies, 19 being carcinomatous, and of these 16 affected the liver.

*Comment.*—In his 1932 paper, Fischer stated that he had performed 412 of 561 necropsies carried out in the period 1928 to 1931. In a subsequent paper (*51*), he states that as Medical Research Officer to the Rand Mines, Ltd., he carried out all autopsies from August 1926 to September 1939. The discrepancy is difficult to explain although Berman (personal communication) has suggested that the clinicians may have signed some of Fischer's necropsy reports. If this is so, Fischer's series of necropsies will have the added advantage of both consistency and validity of diagnostic criteria since a trained pathologist was involved.

Fischer's papers strikingly demonstrate the differences between the East Coast and Union natives as regards their liability to schistosomiasis, multilobular cirrhosis of the liver, and hepatoma. He did not give the proportion of East Coast natives in mine employ, but from 1928 this was controlled by the Mozambique convention (*52*) and was gradually reduced. In the second series he quotes 326 consecutive necropsies, 57 percent of which were on East Coast miners. From figures supplied to me by the Transvaal and Orange Free State Chamber of Mines it is evident that, in general, at this time East Coast natives represented less than 50 percent of the Transvaal Bantu miners, and the preponderance of cancers in East Coast natives referred to in Fischer's first series cannot be explained by a relative preponderance of these laborers in the mining population.

In the assessment of the prevalence of schistosomiasis it must be borne in mind that the actual rates of infection are much higher than macroscopic appearances indicate. Higginson and De Meillon (*53*) have shown by digesting with 10 percent potassium hydroxide rectum, bladder, and liver obtained at Necropsy that *Schistosoma haematobium* ova can be recovered in 26 percent of Union natives, although previously only 1 percent of this population has been reported as showing macroscopic schistosomiasis. In cases of hepatoma, the ova were recoverable in 27 percent, so that no significant correlation was demonstrable between the 2 conditions. One may conclude, therefore, that the real prevalence of schistosomiasis in the East Coast miners must have been exceedingly high indeed, and it is probably not without significance that it was in this group that the 2 cases of carcinoma of the bladder were detected.

In 1934 Strachan (*54*) published a report on malignant diseases diagnosed at 3,851 necropsies over the 10-year period 1924–33 at the Johannesburg General Hospital. Of these, 1,901 were on Bantu subjects, among whom the greatest occurrence of cancer was in the age group 30 to 39, as compared with 50 to 59 in the comparable European group. Ninety-one cases of malignant disease were found in Bantu patients, 73 being car-

cinomas, including 37 hepatomas (52%). Hepatoma was found in 2.43 percent of Bantu male cadavers, and in 0.43 percent of female—a ratio of 6:1. Hepatoma occurred at a relatively early age and constituted 25 out of the 36 carcinomas found in Bantu under the age of 40, *i.e.*, about 70 percent. Schistosomal lesions were found in 2 of the 37 hepatomas, and cirrhosis was definite in 30, which Strachan suggested, "may be of aetiological significance."

*Comment.*—Strachan's figures, though subject to the defects of all necropsy statistics, are important because in an urban population not in mine employ (though it may have contained some ex-miners) they demonstrate a high proportion of hepatomas, a sex ratio independent of mere population differences, and an association with cirrhosis apart from obvious schistosomiasis. He does not say how many diagnoses were confirmed histologically, but at this time histologic confirmation was not invariably employed, according to Dr. B. J. P. Becker, assistant to Professor Strachan from 1936 onward (personal communication). This receives confirmation in Berman's case histories (*9*) and in Berman's Table V (*55*), from which it is apparent that microscopic confirmation was lacking in 28 out of 30 necropsies carried out at the Johannesburg General Hospital on Bantu patients with hepatoma. Strachan noted that differences in the carcinoma/sarcoma ratio between European and Bantu patients were largely accounted for by the age distribution of the population, and it is clear that the Bantu population studied here was a young one and therefore less liable to tumors of old age.

In 1935 Berman (*8*) published the first of a series of papers, which, from the large numbers involved and the striking nature of the cancer ratios derived, threw into prominence the frequency of hepatoma in the Bantu. He examined 2 distinct groups of patients, the first being an urban Bantu population admitted to the Johannesburg Non-European Hospital (a section of the General Hospital of this city) over the 8-year period 1926–33. The second group was drawn from the mine hospitals on the Witwatersrand from 1925–33.

The population, he noted, was not normal for age and sex, this being most obvious in the miners, who were all males "whose ages vary between 18 and 45 years, the majority being about 30 years." (p. 15). He emphasized the difficulty of age assessment in the unsophisticated tribal Bantu (p. 21).

In the Johannesburg urban population, 267 cases of malignant disease were diagnosed, 181 of these diagnoses being based on necropsy, histologic diagnosis, operation, or radiography. During this period there were 41,920 in-patients, and the Bantu population for Johannesburg numbered 148,000. The site was given in 215 cases and hepatoma was found in 41 cases, 36 males to 5 females. In these cases where the site was stated, therefore, hepatoma represented 31 percent of 116 cancers in males and 5.1 percent of 99 cancers in females.

Among miners, 270 cancers were diagnosed, 253 (93.7%) being carcinomas; of these 229 were hepatomas (90.5% of carcinomas). The diagnosis was based on necropsy in 170 cases (*i.e.*, in 67.2% of the carcinomas). In the East Coast Bantu the carcinoma rate was almost 6 times that of Union natives.

The urban population gave a total cancer rate of 22.6 per 100,000, according to the Johannesburg Medical Officer of Health, for the years 1926–32 (this figure, however, referred to deaths only and included miners). The miners showed a rate of 15 per 100,000, and Berman concluded, therefore, "that the relative incidence of malignancy in the semi-civilised Bantu of both sexes and all ages in the urban area of Johannesburg differs very little from the incidence of this disease in the selected primitive aborigine working on Witwatersrand Gold Mines." (p. 16).

*Comment.*—Striking and valuable as these figures are, they cannot be accepted without qualification. The records of the non-European hospital were certainly grossly inadequate, as Berman has told me, and my own experience with this hospital a few years later confirms this. Furthermore, many of the patients at this hospital would have been visitors from outside districts and were not all comparable to the Johannesburg Bantu population. In a more recent survey of Johannesburg hospitals, for example, 44 percent of the Bantu cancer patients were visitors (*10*). Berman drew attention to other distorting factors—the younger males preponderate and the chronically ill tend to go home. It is, therefore, unwise to attach quantitative significance to the crude rates derived from these figures.

Berman's studies of clinical cases covered 8 of the 10 years embraced in Strachan's necropsy studies of the same hospital. Comparison with Strachan's statistics provides an interesting demonstration of the limitations of necropsy studies. Whereas Strachan recorded 37 hepatomas to Berman's 41, many other cancers appear much less frequently at necropsy. Among these were uterine cancers (4 in the necropsy series to 42 in the clinical series), stomach (6 to 23), breast (2 to 25), skin (1 to 16), and buccal cavity (3 to 11). Their figures are identical for cancer of the esophagus (3), urinary bladder (5), and lung (0). Strachan's necropsy figures, for example, give a hepatoma/carcinoma ratio twice that reported by Berman, *viz.*, 62.5 percent (35/56) for males and 11.75 percent (2/17) for females, as compared with Berman's clinical ratios of 31 percent (36/116) and 5 percent (5/99), respectively. Clearly, necropsy studies miss many cancers accessible to clinical diagnosis—a fact often emphasised but as often ignored.

Certainly this series does not confirm Steiner's conclusion (p. 20) that "In series of tumors diagnosed by unselected necropsy, the most accurate representation possible is obtained." If discharge from the hospital is, for any reason, selective, *i.e.*, affected one way or the other by such factors as the rate of a patient's course downhill, shortage of beds, desire to die at home, particular medical interest, or difficulties of nursing, then, no matter how unselected the necropsies are, the population of cases dying in the hospital has already been incalculably modified.

Berman also noted the high frequency of female genital cancer. In this series, uterine cancers (42 cases) outnumbered hepatomas (41), forming 42.5 percent of female carcinomas.

On his own cases, then numbering 25, Berman himself performed the necropsy, with histologic confirmation in all, but for the rest he had to rely on the reporting of others, where the same safeguard could not be maintained. Furthermore, all lungs are sent to the Silicosis Bureau (which does not report to the mine any lung cancers detected in its scrutiny of the lung tissue).

In these circumstances, where necropsies are performed by persons without special training in pathology, one would expect that few hepatomas would be missed, as the enlarged liver would be a striking feature as soon as the body was opened. Tumors of other sites, *e.g.*, of testis, bronchi, or brain might be more easily missed, and the result would be to influence greatly the

proportion of hepatomas to other neoplasms, although the hepatoma rate would be unaffected.

In 1 group of mines (*56*) the accidental-death rate over 20 years averaged 3.109 per 1,000 miners (his Table III) out of a total death rate of 10.618 (his Table II). Necropsies on the cases of accidental and sudden deaths are carried out at Medico-Legal Laboratories, and latent cancers encountered here are reported to the mine (*57*) so that cases of hepatoma are not lost from this source.

Des Ligneris (*58*) has noted certain other factors likely to favor a relative preponderance of hepatoma in mining series, *viz.*:

1. Age. Other cancers are relatively rare in the age groups represented, whereas, in the Bantu, hepatoma has an early onset. Carcinomas, especially, would be rare in such a population.
2. Sex. Hepatomas are commoner in males.
3. Methods and terms of recruitment. The native miners are obliged to pass severe medical tests, so that no obvious wasting disease would be allowed to pass; this tends to exclude other cancers. Pearson (*59*) has described the recruiting procedure. The recruit in fact undergoes 3 medical examinations before he can start work as a miner, *viz.*, at or near his place of residence, at the Witwatersrand Native Labour Organization Hospital in Johannesburg, and at the mine to which he is allotted.

To these may be added certain other influences such as:

4. Disposal. Sick miners are often repatriated if fit to travel, *e.g.*, with tuberculosis [(*56*), p. 54] and with hepatomas (*60*), so that fulminating diseases are more likely to appear at necropsy. Clinical diagnoses, of course, were accepted by Berman, and it should be remembered that mine medical officers are acquainted with the clinical features of this condition. In Berman's (*2*) series of 75 cases of hepatoma in miners, however, 12 (16%) were discovered accidentally in patients whose illness was due to unrelated conditions; these presumably might have been missed in repatriates.
5. The miner stays on the mine for a limited period, and is then repatriated. This tends to eliminate conditions of slow development, and calculations of person-years of exposure are likely to overestimate the time period, even for conditions as acute, relatively, as hepatoma (*10*). Berman gives the period of service as rarely longer than 9 months [(*2*), p. 7]. The General Manager of the Native Labour Organizations of the Transvaal and Orange Free State Chamber of Mines, however, stated in a letter to the author that:

> ". . . the average length of time spent on the mines by East Coast and Tropical Natives in any one period of service be regarded as eighteen months. B. S. A. Natives [*i.e.*, from the Union and British Protectorates] are not subject to compulsory repatriation and may remain in mining employ for as long as they wish before returning home. The actual periods spent on the mines by B. S. A. Natives accordingly show considerable variation, but, it may be mentioned that, in 1943 it was estimated that the average Native mineworker from the Cape Province stayed fourteen months on the mines in each period of service. In 1951, it was estimated that, on the average, B. S. A. Natives were spending between ten and eleven months on the mines."

The miner's contract, however, may be for a period of 1, 3, 6, 9, 10, or 15 months, and consequently the length of stay of an individual may be very much less than the average duration.

The total absence of cancer of the skin and stomach from Berman's series is quite as striking as the incidence of hepatoma. Furthermore, in this series, representing 1,800,000 patient-years between the ages 18 to 45, only 1 tumor of the testis (sarcoma) is recorded. From age-distribution figures supplied to me by the Transvaal and Orange Free State Chamber of Mines, table 2 shows that 86 would have been expected from a similar population of Danish males using Clemmesen and Nielson's figures for the number of testis tumors, including seminomas, detected from death certificates and hospital cases in the entire Danish population for 1943–47 [Table IVG (*61*)].

TABLE 2.—*Tumors of the testis expected in Berman's population of miners, assuming Danish rates**

| Age group | Danish rate per 20,000 (*percent*) | Proportion of total mining population (*percent*) | Proportion expected (1,800,000 miners) (*percent*) |
|---|---|---|---|
| 0–29 | 0. 9 | 0. 6118 | 49. 6 |
| 30–39 | 1. 2 | . 3040 | 29. 2 |
| 40–49 | 1. 0 | . 0779 | 7. 0 |
| 50–59 | 0. 4 | . 0063 | . 226 |
| Total | | | 86. 03 |

*Number actually reported: 1.

Three carcinomas of the lung are reported by Berman (*8*)—a rate of 0.17 per 100,000. Unfortunately, the reports on the Miners' Phthisis Medical Bureau, which deals with all lungs including those from medico-legal necropsies, do not distinguish primary from secondary carcinoma of the lung and so are not helpful. This distinction was made in the Annual Reports of the South African Institute for Medical Research (*62*) for the period 1948–53 inclusive, however, when 8 primary carcinomas of the lung (confirmed histologically) were reported in 3,966 specimens from Bantu miners. This represents a necropsy ratio of 0.2 percent. The death rate for 1 group of mines has been given as 10.6 per 1,000 (*56*), from which one may generalize that if these figures can be applied to all miners, an over-all lung-cancer rate of 2 per 100,000 obtains for this later period—nearly half that reported for liver cancer in Union miners.

Berman confirmed Fischer's evidence that miners do not form a homogeneous group and that East Coast natives were far more liable to carcinoma and sarcoma than South African natives. If these groups are not distinguished, figures derived from miners, or from groups that include miners, will reveal hepatoma rates or ratios that may vary as much as 600 percent, according to the proportion of East Coast Bantu represented. The similarity that Berman noted between cancer incidence in the Johannesburg urban Bantu and the general mining population was, therefore, fortuitous and it is not legitimate to treat such figures as though they are comparable.

In 1935, as an appendix to Berman's study (Tables XXI and XXII) (*8*), further details from Lovedale Hospital were provided by MacVicar. From May 1925 to May 1933, 7,672 in-patients provided 65 cases with cancer, of which 50 were carcinomas. Thirteen hepatomas were encountered, 9 being in males (hepatoma/carcinoma ratio 9:27 or 33⅓%)

and 4 in females (hepatoma/carcinoma ratio 4:23 or 17.4%). The average age for these patients was 52.

*Comment.*—This series provided a remarkably high average age for the hepatoma patients and also a relatively high number of female cases. The diagnostic methods employed are not mentioned in this short note.

After des Ligneris left Elim Hospital, to become a research fellow at the South African Institute for Medical Research, he continued to examine the statistics of this hospital, and in 1936 (*7*) he published figures for the cancers seen over the period 1925–36. A marked increase in the ratio of carcinoma to sarcoma was found. He did not mention secular changes in the hospital population, which might well have been occurring (*63*) and which are well-known, to explain the carcinoma/sarcoma ratio (*64,65*). In 12,373 patients, 84 cancers were found, representing 31 sarcomas (including melanomas) and 53 carcinomas (including 3 hepatomas, 11 of the cervix uteri, 10 of skin, and 5 of stomach). Most of these cases were examined histologically.

Des Ligneris also gave figures for 3 other Swiss mission hospitals in Mozambique, roughly 400 miles southeast of Elim. Chikhumbane lies in the Limpopo Valley near Chai-Chai (alias Vila de João Belo), latitude 25.2′30″ S, longitude 33.40′ E. Here, during 1928–31, 5,800 patients were treated and 55 cancers diagnosed—10 sarcomas, 40 carcinomas (including 14 hepatomas and 15 carcinomas of the bladder), and 5 mixed tumors of the salivary glands.

At Lourenço Marques for the period 1934–35, 7,772 patients were treated and 2 sarcomas and 23 carcinomas (9 hepatomas, 13 uterine, and 1 breast) were diagnosed.

*Comment.*—In this factual and balanced paper des Ligneris commented on the striking difference between adjoining subtropical areas and warned against general conclusions from local studies. He answered the possible criticism that Bantu patients suffering from cancer would not come to the hospital and would rather consult their medicine man by remarking that it is unlikely that they would stay away with cancer, since they come in large numbers when suffering from tuberculosis. On the grounds of the geographic variation demonstrated, he doubted the relationship between hepatoma and skin pigmentation, then suggested by Berman; he postulated that liver cancer was due to a factor affecting the patient in his childhood days and was not an influence encountered during the period of work on the mine. As schistosomiasis is common in the Zoutpansberg district served by Elim Hospital, he concluded it was doubtful whether the main cause of hepatoma could be schistosomiasis, especially since the population of countries like Egypt, which have a much greater incidence of schistosomiasis, do not display the same tendency to hepatoma.

Des Ligneris also quoted figures given him by Dr. F. S. Simson, of the same Institute, showing that in the 7-year period 1928–34, "out of 114 specimens of primary liver cancer which were examined by him, not more than 6 were from European cases."

In 1940 (*66*), Berman re-examined his earlier figures for hepatomas. Age distribution of hepatoma cases was calculated for a group of 144 Bantu cases, 103 male miners, and 41 of mixed sex and age drawn from the

urban population. The tribal distribution was also given, but as that of the parent population was omitted it is difficult to draw any conclusions from these figures other than that hepatoma may occur at an early age and that certain tribes are affected.

The clinical features of the disease in the Bantu were described by Berman in 1940 (*55*) and again in 1944 (*67*), based on records of 66 cases, of which 25 had been miners in his personal care and 41 were cases from the Johannesburg Hospital. These demonstrated an average stay in the hospital of 18.2 days, the maximum being 81, which indicates the fulminating nature of the condition. He did not distinguish between the different clinical types of hepatoma nor between those who died in the hospital (61 cases) and those who were discharged (5 cases) (*9*), but this does not make much difference for the crude length of stay. Fifteen percent of the cases were classified as "occult," *i.e.*, the cancer was diagnosed accidentally during the treatment of other conditions or at necropsy when death was due to other causes.

In 1940 Prates (*68*) analyzed the records of hepatomas seen at the Miguel Bombardo Hospital in Lourenço Marques during the years 1930–38. Eighty-five cases were recorded from 49,000 patients, a proportion double that reported for the non-European hospital at Johannesburg. Ages ranged from 13 to 55 years, most being under 40; there were 80 cases in men to 5 in women.

> *Comment.*—These figures were preliminary, being produced after little more than 2 months' study. The diagnostic methods employed are not mentioned nor the age and sex distribution of the hospital population. Prates considered that the tumors were more frequent in the south of the Mozambique colony, the area from which most miners were drawn at that time. He discussed the likelihood of toxic substances being present in alcoholic beverages, saying "In my cases I succeeded in ascertaining that some patients drink daily 10 litres of a beverage obtained by the fermentation or distillation of mealies (Buputo) and about 3 litres of a strongly alcoholic drink obtained from the fermentation and distillation of caju peduncles and manioc tubers." (p. 99). This habit, however, is not found in the Union, and will not explain the relative frequency of hepatoma among the Union Bantu.

In 1948 Prates (*69*) reported 70 additional cases of hepatoma, all of the hepatocellular type. Whereas in his first paper he had drawn attention to the native beverages taken by the population there, in this publication he tended toward schistosomiasis as the cause, although he stated that animals fed on native drinks showed toxic degeneration and proliferative lesions in different organs.

Berman (*11*) gave figures of hepatomas in all Bantu miners for the 20-year period 1925–44, when 826 cancers were diagnosed, of which 772 were carcinomas—670 of these being hepatomas, (*i.e.*, hepatomas formed 86.8 percent of the carcinomas and 81 percent of the total cancers). The average age of the population was given as 30 years, and the annual size of the population, 200,000 to 300,000. East Coast natives comprised 35 percent of the miners and contributed 78.8 percent of the 670 hepatomas (507). Hepatoma thus was 6 times as frequent in the East Coast Bantu

as in the Union Bantu miners. The liver was invariably the seat of multilobular cirrhosis.

Berman also gave the age distribution in 402 of the 670 hepatomas. Since a few cases are reported in the group 51 to 60 years it is clear that the age limits (18 to 45 years) given for miners were a rough approximation only.

Berman drew attention to the fact that the East Coast natives suffered more often from parasites and that milk was less plentiful in their places of origin.

The results of these studies were incorporated in his book published in 1951 (*2*). Here he quoted the rates in the 2 racial groups of miners, as 5 per 100,000 in Union natives, and 29 per 100,000 in Mozambique natives (Fig. 2, p. 10). The frequency of hepatoma to all carcinomas is given in Table VI as 50.9 percent (p. 8) and the age distribution of hepatoma in Table XII, which is based on the ages of 402 miners and 41 urban patients drawn from the 1935 study.

*Comment.*—These valuable records have led to confusion because they included population groups that are not comparable in age, sex, or diagnostic method. Thus in [Table VI, (*55*)], 2,796 carcinomas and 1,425 hepatomas are quoted from the Bantu races of Africa, but the results are heavily weighted by the mining population, in whom, as we have seen, the bias is strongly in favor of hepatoma. There are 12 references to South African sources, 4 of which include miners (Pirie, Orenstein, Berman, and the Johannesburg M. O. H. reports). At least 837 carcinomas and 720 hepatomas were derived from miners.

Several sets of patients are counted twice in this table, as well as in its predecessor [Table V, (*55*)], for Berman's own figures must include those quoted by Orenstein and many of those given in the reports of the Johannesburg Medical Officer of Health.

Reduplication also occurs in the urban cases of hepatoma, since the reports of the Medical Officer of Health for Johannesburg record all deaths in the Johannesburg population and would therefore include some of Pirie's cases, diagnosed at the Institute for Medical Research in this city, as well as all deaths in Beyer's and Berman's series from the Johannesburg Hospital. Berman omitted certain small series of cancers in which hepatoma is not mentioned, *e.g.*, that of Casalis, but these omissions do not noticeably affect the figures quoted.

If one separates the urban and mining populations and omits the records of the Medical Officer of Health, one can derive table 3 for purposes of comparison. It has been necessary to omit Pirie's figures, as miners were not separately distinguished, and to include Berman's hospital series in the urban group.

The effect of weighting cancer figures with miners is also demonstrated in Berman's own papers. The inclusion of a greater number of miners in Table VI of Berman (*2*), for example, has caused the percentage of hepatomas to rise from 37.4 [Table V, (*55*)] to 50.9. Similarly, the age distribution [Table XII, p. 19 (*2*)] shows a distinct shift to the younger group as compared with that derived from 41 hospital cases [by subtracting the miners given in Table V, (*11*)]. In the mining group 11 percent were 41 years and over, as compared with 34 percent in the hospital group.

The age distribution is given for 402 hepatomas among miners [Table V of Berman's 1950 (*11*) paper]. In the following discussion it should be remembered that, as Berman has emphasized [(8), p. 21], these miners have

TABLE 3.—*Earlier South African hepatoma/carcinoma ratios*

| | Carcinomas | Hepatomas | Ratio (*percent*) |
|---|---|---|---|
| *Non-mining cases* | | | |
| Macfarlane (*42*) | 35 | 7 | 20. 0 |
| MacVicar (*43*) | 97 | 22 | 22. 7 |
| MacVicar (*8*) | 50 | 13 | 26. 0 |
| Watkins-Pitchford (*20*) | 25 | 2 | 8. 0 |
| Beyers (*46*) | 75 | 22 | 29. 4 |
| Des Ligneris (*44*) | 21 | 0 | 0 |
| Rosset (*62*) | 53 | 3 | 5. 6 |
| Berman (*9*) | 215 | 41 | 19. 1 |
| | *356* | *110* | *19. 3* |
| *Miners* | | | |
| Berman (*11*) | *772* | *670* | *86. 8* |
| Union miners (65%) | | 163 | |
| Mozambique miners (35%) | | 507 | |

very little idea of their age, and Stibbe (*70*) has felt justified in classifying them into 3 groups only: "Youthful, or immature—probably under twenty. Adult—say between twenty and thirty. 'Madala,' or old—over thirty." Calculations based on such estimates of age are therefore only rough approximations. One must assume that 268 cases whose ages were not given did not differ significantly in age and race from those whose ages are known. If, however, hepatoma occurs at an earlier age in East Coast natives, and these tend to be more illiterate than Union natives, then their omission would affect the apparent age distribution. Assuming that the 402 cases are truly representative, and that the age distribution given by the Chamber of Mines is applicable to both groups, we can calculate (table 4) the annual rates per 100,000 in the age groups of the population from the formula:

$$\text{Number of cases of hepatoma in group} = \frac{x}{pyt} \times 100{,}000$$

(where $x$ is the number of cases encountered in an age group and $p$ the proportion of the total population ($y$) found in this age group during $t$ years.)

These figures indicate that although hepatoma manifests itself at an early age, it is not limited to the young and its incidence increases with age. Secondly, the figures are higher than those obtained in the American Negro population—those given here were based on the resident cancer-incidence rates for the United States, 1947–48 [Dorn and Cutler, Table IVc, (*71*)]. As the age groups in the American series were centered on units of 10 years, I have taken an average for 2 adjacent decades, ignoring the minor effect of changing numbers in the different age groups.

Berman's statistics related to the combined group of Union and Mozambique natives, and he derived separate rates from the proportions of the average annual populations. I have calculated these from figures submitted by the Native Labour Organizations of the Transvaal and Orange Free State Chamber of Mines. One hundred sixty-three hepatomas were found in a total average annual population of 3,521,698 Union natives (4.63/100,000 per annum) and 507 cases in a total average annual population of 1,647,661 tropical and Mozambique natives (30. 8/100,000 per annum). From these figures the rate for tropical and Mozambique natives appears to be 6.65 times that of the Union natives.

TABLE 4.—*Estimated age-specific hepatoma rates from Berman's figures (mixed mining population, i.e., Union and Mozambique Bantu)*

| Age group | Hepatomas ($x$) | Proportion ($p$) | Rate/100,000 per annum | Corrected for those of unknown age | American Negro (*66*) |
|---|---|---|---|---|---|
| 11–20 | 22 | .1272 | 4.5 | 7.4 | 0.0 |
| 21–30 | 196 | .4846 | 10.4 | 17.3 | 0.0 |
| 31–40 | 140 | .3040 | 11.9 | 19.8 | 2.4 |
| 41–50 | 41 | .0779 | 13.5 | 22.6 | 9.3 |
| 51+ | 3 | .0063 | 12.3 | 21.0 | 16.4 |

Age-specific rates (table 5) can be derived if one assumes 1) identical age distributions of the Union and East Coast mining populations and their hepatoma patients (which is unlikely, since a stronger carcinogenic stimulus is likely to manifest itself at an earlier age), and 2) that hepatoma is diagnosed with equal facility in both groups—*i.e.*, that the proportion of cases missed by reason of selection, repatriation, length of stay, or accidental death is the same.

These figures can be calculated (table 5) by deriving a coefficient of distribution from the proportional distributions of hepatomas in miners. [Union natives provide 24.3% of hepatomas, while representing 68.127% of the population. The coefficient (proportion of hepatomas to proportion of population) for Union natives is 0.357 and for Mozambique natives 2.34.]

TABLE 5.—*Estimated age-specific rates for Union and Mozambique mine Bantu*

| Age group | Berman's mixed group corrected for those of unknown age | Union | Mozambique |
|---|---|---|---|
| 11–20 | 7.4 | 2.60 | 17.3 |
| 21–30 | 17.3 | 6.18 | 40.5 |
| 31–40 | 19.8 | 7.08 | 46.4 |
| 41–50 | 22.6 | 8.09 | 53.0 |
| 51–60 | 21.0 | 7.51 | 49.2 |

A comparable group of young males was studied by Findlay (*72*), who reported on 227,000 West African forest Negro soldiers, 18 to 40 years of age. Younger ages predominated and two thirds were under 25 years. Between 1940–45 inclusive, 60 malignant tumors were diagnosed of which 37 were hepatomas. The incidence of malignant tumors was reported as 26.4 per 100,000.

*Comment.*—Unfortunately Findlay's figures represent ratios only, as the precise time period is not clearly stated. If the entire population were at risk for the whole period of 5 years, the annual rates would be one sixth of those given. The hepatoma/carcinoma ratio was 68.5 percent, and the hepatoma/cancer ratio 61.5 percent.

Higginson (*73*) surveyed the site distribution in a histologically confirmed series of 500 cancers at Baragwanath Hospital, Johannesburg. In this series, which included males and females of an urban population

and excluded miners, both biopsies and necropsies were used. The hepatoma/carcinoma ratio was 5 percent and the hepatoma/cancer ratio 4 percent.

*Comment.*—Older age groups were somewhat better represented in the Johannesburg urban population in 1951 as compared with 1925; the sexes were about equally represented, whereas in 1925 the male/female ratio was 5.5 to 1 (*32*). The male hepatoma/carcinoma ratio in Higginson's series was 10.45 percent (18/172) and the hepatoma/cancer ratio 7.56 percent (18/238). The corresponding female figures are 0.91 percent (2/220) and 0.765 percent (2/262). Higginson's paper also gives the percentage of cases in which histologic confirmation was lacking, and thus provides a useful index of the commoner errors of interpretation.

A site-type survey of certain cancers diagnosed histologically at the South African Institute for Medical Research during the period 1949–53 (*74*) is less informative, as the population is not as easily definable, but the figures may be worth quoting: 275 hepatomas were diagnosed in non-Europeans compared with 9 in Europeans—a ratio even more striking than the figures given by Simson, quoted by des Ligneris. For the particular regions dealt with in this paper, the hepatoma/carcinoma ratio in non-Europeans was under 11 percent (275/2,509).

Dr. H. Garin (*75*) has also sent me figures from the Swiss Mission Hospital at Lourenço Marques for the years 1933–43, inclusive. These figures included the 2 years reported by des Ligneris (*7*) for whose data they provide valuable corroboration. The standard of diagnosis was high, radiology was employed, all cases dying in the hospital underwent necropsy, and histopathologic diagnosis was available. Dr. Garin carried out these examinations himself as a rule, using frozen and paraffin sections, and consulted des Ligneris where necessary. Liver abscesses were diagnosed at operation and by exploratory puncture. In 1 or possibly 2 cases the tumor was diagnosed by needle biopsy.

These figures are given in table 6, while table 7 gives the diagnoses of patients seen at the Dispensary and Hospital of the Swiss Mission, Lourenço Marques.

*Comment.*—It will be noticed that amebiasis was very prevalent, and liver abscesses were diagnosed nearly 3 times as often as hepatoma. Syphilis and symptomatic vesical schistosomiasis were extremely common indeed.

Hepatoma and uterine carcinoma are very prevalent in this general population. In the males, hepatoma represented over 56 percent of the total cancers and 68 percent of the carcinomas. Among females, hepatomas formed 24 percent of cancers and 28 percent of carcinomas (as compared with uterine-cancer ratios of 49 and 57 percent, respectively).

A notable feature of this series is the sex ratio of these hepatoma ratios—for the hepatoma/cancer ratio this is 2.3 to 1 and for the hepatoma/carcinoma ratio, 2.4 to 1. These figures suggest the highest relative frequency of hepatoma in Bantu females yet encountered. Nevertheless, male hepatoma patients are concentrated in the earlier age groups as compared with the female cases, who are older. This may indicate a more potent carcinogenic stimulus in males.

TABLE 6.—*Malignant tumors seen at the Swiss Mission Hospital, Lourenço Marques, from 1933–43, inclusive**

| | (1) 0–19 | | (2) 20–29 | | (3) 30–39 | | (4) 40–49 | | (5) 50–59 | | (6) 60+ | | Total cols. 1–6 | | (7) Indians | | (8) Europeans | | Total cols. 7, 8 | | Grand total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F | |
| *Carcinomas* | | | | | | | | | | | | | | | | | | | | | |
| Kidney | | | 1 | | 1 | | | | | | 1 | | 3 | | | | | 1 | 3 | 1 | 4 |
| Bladder | | | 2 | 1 | 2 | 2 | 3 | 2 | | 1 | | | 7 | 6 | 1 | | 1 | 1 | 9 | 7 | 16 |
| Prostate | | | | | | | | | | | 2 | | 2 | | | | 2 | | 4 | | 4 |
| Mouth and lips | | | | | | | | | | | 1 | | 1 | | | | 1 | 2 | 2 | 2 | 4 |
| Esophagus | | | | | | | | | | | | | | | | | 2 | | 2 | | 2 |
| Stomach | | | | | | | | 1 | | | 1 | | 1 | 1 | 3 | | 5 | 1 | 9 | 2 | 11 |
| Intestine | | | | | | | 2 | 1 | | | | | 2 | 1 | | | 3 | 2 | 5 | 3 | 8 |
| Liver | 3 | 1 | 21 | 6 | 14 | 5 | 4 | 5 | 5 | 2 | | 2 | 47 | 21 | | | 2 | | 49 | 21 | 70 |
| Spleen and pancreas | | | | | 1 | | | | | | 1 | | 2 | | | | | | 2 | | 2 |
| Larynx | | | | | | | | | | | | | | | 3 | | 1 | | 4 | | 4 |
| Salivary glands | | | | | | | | | | | 1 | | 1 | | | | | | 1 | | 1 |
| Skin | | | | | 2 | | 1 | 1 | | | | | 3 | 1 | 2 | | 4 | | 9 | 1 | 10 |
| Breast | | | | | | 1 | | 1 | | | | | | 2 | | 4 | | 6 | | 12 | 12 |
| Uterus | | | | | | 5 | | 17 | | 13 | | 7 | | 42 | | 1 | | 6 | | 49 | 49 |
| Chorio-epithelioma | | | | | | | | | | | | | | | | | | 1 | | 1 | 1 |
| *Sarcomas* | | | | | | | | | | | | | | | | | | | | | |
| Lymphosarcoma | | 2 | | | | | | | | | | | | 2 | 1 | | | 1 | 1 | 3 | 4 |
| Bone sarcoma | 1 | | 1 | 1 | | | 2 | 1 | | 1 | | | 4 | 3 | | | 1 | 1 | 5 | 4 | 9 |
| Maxilla | 1 | 1 | | | 3 | 2 | 1 | | 2 | 1 | 1 | 1 | 8 | 5 | | | | 1 | 8 | 6 | 14 |
| Uterus | | | | | | | | | | 1 | | | | 1 | | | | | | 1 | 1 |
| Stomach and peritoneum | | | | | | | | | | 1 | | | | 1 | | | | | | 1 | 1 |
| Ovary | | | | | | | | | | | | | | | | | | 2 | | 2 | 2 |
| Retina | 1 | | | | | | | | | | | | 1 | | | | | 2 | 1 | 2 | 3 |
| Brain | | | | | | | 1 | | | | | | 1 | | | | | 1 | 1 | 1 | 2 |
| Total | 6 | 4 | 25 | 8 | 23 | 15 | 14 | 29 | 7 | 20 | 8 | 10 | 83 | 86 | 10 | 5 | 22 | 28 | 115 | 119 | 234 |

*M=male, F=female.

TABLE 7.—*Diagnosis at Dispensary and Hospital of the Swiss Mission at Lourenço Marques, 1933–43**

| | Natives | | | | Indians | | | Europeans | | | Grand total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | C | Total | M | F | Total | M | F | Total | |
| Malignant tumors | 83 | 86 | — | 169 | 10 | 6 | 16 | 22 | 28 | 50 | 235 |
| Benign tumors | 23 | 194 | 12 | 229 | 2 | 11 | 13 | 4 | 39 | 43 | 285 |
| Tuberculosis—lungs | 305 | 110 | 13 | 428 | 111 | 13 | 124 | 84 | 32 | 116 | 668 |
| Tuberculosis—other | 134 | 32 | 7 | 173 | 21 | 3 | 24 | 19 | 8 | 27 | 224 |
| Elephantiasis | 57 | 5 | — | 62 | 7 | 1 | 8 | 1 | 1 | 2 | 72 |
| Liver abscess | 135 | 57 | — | 192 | 9 | — | 9 | 3 | 1 | 4 | 205 |
| Syphilis | 1, 504 | 929 | 168 | 2, 601 | 159 | 31 | 190 | 146 | 63 | 209 | 3, 000 |
| Schistosoma (vesic.) | 1, 469 | 479 | 42 | 1, 990 | 9 | 7 | 16 | 11 | 2 | 13 | 2, 019 |
| Id. rectal | 59 | 10 | 8 | 77 | 2 | — | 2 | 1 | — | 1 | 80 |
| Dysentery (amebic) | 683 | 553 | 208 | 1, 444 | 46 | 22 | 68 | 41 | 24 | 65 | 1, 577 |
| Total | 4, 452 | 2, 455 | 458 | 7, 365 | 376 | 94 | 470 | 332 | 198 | 530 | 8, 365 |

*M=male, F=female, and C=colored.

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Other features of this series include a high frequency of bladder cancers (7.7%), in which sex incidence is equal; probably these can be attributed to the frequency of schistosomiasis (*74*). Uterine cancers are very common (49% of female cancers). "Maxillary sarcoma" is strikingly common (7.1%), but one wonders how many carcinomas of the antrum fall into this category. Carcinomas of stomach, skin, and breast are rare. The frequency of bladder cancers in East Coast miners has already been noted by Fischer (*50*) and Berman [(*8*), p. 23].

## Discussion

### *Do the Bantu Display a High Susceptibility to Hepatoma?*

Although the references to hepatoma from South Africa abundantly confirm clinical impressions as to the frequency of the disease, it is still impossible to assign sufficiently precise quantitative meaning to the ratios and rates which have been derived.

Gilliam (*6*) has emphasized the unreliability of ratio studies of hepatoma and attempted to use published data to extract additional information. This attempt was less successful than it deserved to be because certain assumptions were required that can be shown to be false. In the first place he based his calculations on a misprint in the article quoted where the age grouping is given as 28 to 45 instead of 18 to 45 years (*66*). Second, he assumed that within this group the age distribution of this population of miners was comparable with that of an American U. S. population. In fact, the Bantu mining population was predominantly a young one, and Berman [(*66*), p. 56] gives the average age as about 30, as is indicated in table 2 where over 60 percent were under 31 years of age. (The hepatoma rates obtaining in this population are given in tables 4 and 5, which represent another attempt at dissecting the existing information). Gilliam's criticism that Berman's Table I (*66*) showed the presence of patients in the 7th decade, was not valid, as the 114 cases in this table included urban Bantu as well as miners. His conclusion, nevertheless, was correct, that the ages of some miners with hepatoma lay outside the upper limit of 45 years.

In the light of this additional information on Berman's material, therefore, there can be no doubt that the incidence of hepatoma is considerably higher in the Bantu miners than Gilliam estimated.

The conclusion is also confirmed by preliminary examination of the first 2 years of a cancer-rate survey in the Johannesburg Bantu (*76*).

Findlay's rates, which are the only other modern hepatoma figures for African Negroes, refer to West Africans and not Bantu. The lack of a time scale here has been noted earlier, but supposing the figures to refer to the entire population at risk for 6 years, the hepatoma rate was 2.7, which is half that reported by Berman for Union mine Bantu and corresponds to our rate for males in the age group 15 to 24. From Johannesburg Bantu rates, a population similar in distribution to Findlay's, and between 15 and 34 years of age, would have produced over 92 hepatomas in 6 years, as compared with the 37 reported.



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### *Are Bantu Miners More Susceptible than Urban Bantu?*

Peller (*77*) and Lonsdale (*78*), who have regarded the high ratios of hepatoma reported from miners as evidence of unusually high incidence in miners, as distinct from other Bantu, attributed this to carcinogenic conditions obtaining in the gold mines, Lonsdale suggesting radioactivity as the possible carcinogen. The relative immunity of white miners is not explained. The assumption that miners are more susceptible is not supported by the evidence presented here, while the suggestion that miners are exposed to excessive radioactivity seems to be equally baseless. The report of the Miners' Phthisis Medical Bureau (*79*) states, "Investigation has shown that the ore bodies of the Witwatersrand contain only a minute and negligible amount of radio-active substances." (p. 11), this opinion being based on Pirow's (*80*) geological studies.

From evidence derived from a survey of cancer incidence in Johannesburg (*76*) it is apparent that Bantu miners derived from the Union and Protectorates are not more susceptible than Bantu urbanized males in Johannesburg. In fact an urban male population, having the same size and age grouping as Berman's Union miners, would have produced an expected number of 542 hepatomas as compared with the figure of 163 which he reported. One should not regard this difference too seriously since the influences operating on his material would tend toward underestimation of the hepatoma rate and overestimation of the hepatoma ratios. This once again throws into relief the difficulty of interpreting cancer ratios. In Union Bantu miners Berman (*8*, *66*) has found a hepatoma/carcinoma ratio of 87 percent (53 cases in 61). By comparison, urban male Bantu from our Johannesburg survey with apparently more than 3 times the hepatoma incidence showed a hepatoma/carcinoma ratio of 24.6 percent. Nevertheless, the largest Johannesburg Bantu hospital provided a histologically confirmed crude hepatoma/carcinoma ratio in males of 10.45 percent (*73*)—this difference, presumably, being attributable to a greater proportion of patients with other cancers coming from outside districts.

Further evidence should not be necessary to demonstrate the unreliability of crude ratios derived from populations differing widely in age grouping and subjected to ill-defined selective factors.

### *Factors Responsible for Certain Prevailing Views on Hepatoma Prevalence*

The early onset of hepatoma is a characteristic feature of these tumors in the Bantu and is largely responsible for the striking variation in the ratios published. The younger the population, therefore, the higher is the hepatoma/carcinoma ratio likely to be, and simple differences in age and sex composition can, as we have seen, cause the hepatoma/carcinoma ratio to fluctuate from 90 to 5 percent, without any real significance as regards incidence. In an urban population under 45 years, hepatomas represent about 50 percent of all carcinomas diagnosed. Older populations are

associated with a markedly lower hepatoma ratio, even though hepatoma rates increase with age. We found, for example, approximately the same number of carcinomas of the esophagus in males over 45 as hepatomas, and if the diagnostic method is limited to cases diagnosed in the hospital or with histologic confirmation only, then carcinoma of the esophagus was the more common cancer at these ages (*76*). Nevertheless, the frequency of carcinoma of the esophagus passed undetected by clinical observers.

### *The Effect of Age on the Sex Ratio of Hepatoma*

The published sex ratios all show a preponderance of males, but the relative preponderance varies. Both Garin's figures for the Swiss Mission, Lourenço Marques, and ours, however, indicate that the sex distribution of hepatomas varies with age, and that although female cases are rarer in the younger age groups they become relatively more common in the older groups, and the disparity between the sexes tends to disappear.

In Garin's series, male cases were more than 3 times as prevalent as female cases below the age of 40; above this age the numbers seen were equal.

Clearly crude sex ratios of hepatoma are unreliable and age-specific sex ratios are necessary.

### *Racial Causes*

Occasionally this susceptibility to hepatoma has been attributed to racial differences. Berman, for example, in 1940 (*66*) concluded that "primary carcinoma of the liver is a disease which is controlled by racial and not by environmental factors." (p. 68) and noted that it is a common cancer among all pigmented races. In 1941 (*81*) he considered environmental factors as well, while in 1951 (*2*) he concluded that there was strong presumptive evidence pointing to local rather than genetic causes, emphasized the ethnic differences in pigmented races, and concluded that "environmental factors, including cirrhosis-producing and carcinogenic agents, are responsible for precancerous and cancerous states of the liver; and that in man these probably act on a favourably prepared soil, the nature of which is apparently determined largely on a nutritional basis." (p. 132).

The figures presented in this paper confirm that hepatoma is commoner in Bantu races in South Africa, but emphasize that the relative proportions apparently vary markedly from place to place. Many but not all of these variations are explicable on tribal or population differences, and the influence of additional, presumably environmental, influences operating at different intensities within the Bantu population seems inescapable.

The figures presented here will be replaced when results of rate surveys have become available. These ratio studies are pointers which emphasize the need for further studies of hepatoma rates in South African native groups living under widely differing but precisely definable conditions.

Such studies will help in the search for relevant etiologic factors, and will provide that factual discipline without which views on the causation of this disease remain speculative or of only local validity.

**Summary**

1) The literature on hepatoma in the Bantu of southern Africa is critically reviewed.

2) Figures submitted from the Swiss Mission Hospital at Lourenço Marques (Mozambique) are presented, confirming the apparently high relative frequency for hepatoma reported from that city, and indicating also a very high proportion of female cases.

3) It is emphasized that comparison of incidence of cancers—whether in different races, or in the same race at different times—can only be placed on a quantitative basis if the age- and sex-specific rates are known. In view of this, wherever possible, plans for the further collection of cancer ratios should be designed to provide cancer rates.

4) The ratio of the sexes varies with age, being very much higher in the first 5 decades than thereafter.

## APPENDIX: Marginal Errors in Enumeration

The population density of objects scattered randomly over a field is usually estimated by counts of sample areas. With this method the possible effects of the margins of the sample areas must be considered. These influences are well known as bearing on the familiar problem of counting objects in a definite area, but are perhaps less well recognized in the enumeration of cancers within a population in a definite period of time—a process which poses essentially similar problems and requires the same precautions.

Two situations should be distinguished:

1) Where the sample area is defined by arbitrary or imaginary limits in a field of continuous distribution, *i.e.*, where the margins can be regarded as neutral.
2) Where the margins of the sample area are predetermined by an actual discontinuity in the field.

### *Case 1.—Neutral Margins*

This is the simpler situation, as the boundaries do not affect the distribution of the objects to be counted. A familiar example is that of red-cell counting in a hemocytometer, where sample areas are defined by the rulings on the counting chamber. In cancer demography a corresponding situation is the enumeration of cancers in a population of a given size between 2 date lines.

Objects lying near the borders must possess a single previously determined reference point, independent of their position relative to the border,

by which the enumerator may decide whether or not an object falls within the area to be studied. In cell counts, for example, the usual rule is that all cells touching the left border of a square are counted in, all touching the right border are excluded. The reference point is thus the extreme right-hand margin of the cell. Similarly, in recording cancers, although the disease represents a process of varying duration, each case must have a single definite reference date on which it will or will not be assigned to the periods of enumeration. The method of determining the date cannot be the same for all diagnostic classes—compare, for example, a cured cancer and one discovered accidently at necropsy. Differences in these criteria will not affect the results, provided the field is examined on either side of the borders to ensure that the cases are counted in a continuous field.

Apart from these decisions, the boundaries, being arbitrary, exert no influence on the number counted, unless a fluctuation in distribution, *e.g.*, seasonal, occurs that has not been allowed for in the position or width of the belt chosen.

### *Case 2.—Influential Margins*

Pre-existing limitations of the field, on the other hand, may exert an additional influence on the count. A familiar histologic example would be that of counts of objects in histologic sections where the field is bounded by the planes of section. In some instances, although the plane of section will have transected the object, the slice of the material included will be too thin to be detected (*82*). A second example is to be found in automatic counting machines which scan the field with a spot of light or a narrow slit. Objects that, though not completely within the scanning belt, are grazed by it will produce signals that may be registered (depending on the sensitivity of the counting system), and the borders will have the effect of increasing the apparent number counted.

An analogous situation occurs in cancer demography if the period of observation coincides with actual events in the lives of the individuals, *e.g.*, where men come to work on the mines for a definite period of service, and are studied during this interval only. Before employment, mine recruits undergo rigorous selection that eliminates any suspected cancers and all wasting conditions (should these have failed to dissuade the potential miner from applying for employment). Similarly, a miner awaiting discharge after a spell of service may conceal symptoms of illness in his eagerness to leave for home. The period of elimination will vary with recruiting and labor conditions, and with the type of cancer, but in any case the true period during which the miner is susceptible to cancer at the normal rates for his age group and social class will be less than the actual period of service, by the combined marginal elimination periods.

When the period of service is less than the average combined marginal-elimination period for a particular cancer, one would expect to find very few cases of this cancer (only those whose development is more rapid

than average or where the period of stay is of more than average length). Thus in Berman's series no deaths from squamous carcinomas of the skin were reported in the Bantu mining population, as the common predisposing factors—albinism and chronic leg ulcers—serve to exclude potential victims, and the period of stay is too short for the occurrence of fatal skin cancers since early cases would be eliminated in medical examinations.

To demonstrate the real period at risk as distinct from the period of service or observation belt, case incidence could be graphed in succeeding time intervals, *e.g.*, months, to find at which point the distribution reaches a steady level (if there is such a steady level, or if seasonal fluctuation occurs, when this has been compensated for). Only when there is no difference in incidence in the border periods as compared with the central time interval, will it be legitimate to regard the period of stay as synonymous with the period at risk, *e.g.*, one would expect rupture of aneurysms of the circle of Willis to follow this latter pattern. With more protracted diseases, incidence will rise to a plateau at the beginning of the stay and fall off again at the end. Assuming an even rise and fall, the total number of cancers reported from the entire stay might reasonably be treated as if derived from the period between the 50-percent incidence marks.

A second possible technique for detecting the extent of the marginal-elimination period would consist in comparing the ratios of cancer incidence in miners with 2 different lengths of stay, *e.g.*, comparing those with a 9-month period of service with the 15-month group. The ratios will naturally be proportional to the true periods at risk, which will be less than the period of service by a constant border amount.

Suppose the periods of service are $T$ and $nT$, and the marginal-elimination period is $K$, then the ratio $R$ of cases detected in period $T$ to those detected in period $nT$ will be not $T/nT$ but $(T-K)$ $(nT-K)$.

From this one may derive:

$$K=\frac{T(1-Rn)}{1-R}$$

Unfortunately the published material relating to hepatoma in miners does not permit these calculations, although it will be evident that rates calculated by Berman must be somewhat lower than the actual rates. Assuming an average stay of 9 months, and a marginal elimination period of 1 month, for example, the period at risk is 8 months and the true rates will be 12.5 percent higher than the estimated. Since some workers remain for 1 month only, these should be excluded from the calculations before starting, as they will tend to reduce the number estimated still more. For acute cancers like hepatomas these errors, however, will probably be of relatively trivial magnitude, but in more slowly developing cancers the difference between the apparent and the real incidence will be profound. Clearly, cancer ratios in such populations will be very unreliable, since different types of tumors will differ

profoundly in their likelihood of being eliminated by prior selection, *i.e.*, the width of the marginal-elimination zones will be different in each case. The ratio of hepatoma to squamous carcinoma of the skin, for example, would be meaningless unless the ratios are derived from real, not apparent, incidence rates.

## References


(*1*) Leading Article: An expedition in search of the cause of cancer. Lancet 2: 720–721, 1951.

(*2*) Berman, C.: Primary Carcinoma of the Liver. London, H. K. Lewis & Co., 1951, 164 pp.

(*3*) Patwardhan, V. N.: Human malnutrition and liver disease in the tropics. Voeding 16: 223–247, 1955.

(*4*) Steiner, P. E.: Cancer: Race and Geography. Baltimore, Williams & Wilkins Co., 1954, p. 284.

(*5*) Dorn, H. F.: The incidence of primary cancer of the liver in the Negro in Africa and the United States. Schweiz. Z. allg. Path. 18: 648–653, 1955.

(*6*) Gilliam, A. G.: A note on evidence relating to the incidence of primary liver cancer among the Bantu. J. Nat. Cancer Inst. 15: 195–199, 1954.

(*7*) des Ligneris, M. J. A.: Cancer in South African natives. South African M. J. 10: 478–485, 1936.

(*8*) Berman, C.: Malignant disease in the Bantu of Johannesburg and the Witwatersrand gold mines. South African J. M. Sc. 1: 12–30, 1935.

(*9*) ———: Primary carcinoma of the liver in the Bantu races of South Africa. M. D. Thesis, University of the Witwatersrand, 1936.

(*10*) Oettlé, A. G.: Problems of research into diseases of the Bantu. South African J. Lab. Clin. Med. 1: 57–59, 1955.

(*11*) Berman, C.: Primary carcinoma of the liver among the Bantu mine workers of the Witwatersrand gold mines. Proc. Transvaal Mine M. Offrs' Assoc. 30: 13–22, 1950.

(*12*) Hirsch, A.: Handbook of Geographical and Historical Pathology (trans. 2nd German ed. by C. Creighton). London, New Sydenham Society, 1883.

(*13*) Clemow, F. G.: The Geography of Disease. Cambridge, Cambridge University Press, 1903.

(*14*) Livingstone, D.: Missionary Travels and Researches in South Africa. London, John Murray, 1857.

(*15*) Addington Hospital, Durban: Report of the Medical Superintendent for the year ended December 31st, 1901. Pietermaritzburg, Times Printing & Publishing Co., Ltd.

(*16*) ———: Report of the Medical Superintendent for the year ended December 31st, 1904. Pietermaritzburg, Times Printing & Publishing Co., Ltd.

(*17*) Bruce-Bays, J.: Primary malignant growth of the liver. Brit. M. J. 2: 630, 1905.

(*18*) Hawes, C. S.: Three cases of primary malignant disease of the liver. St. Bart's Hosp. Rep. 41: 161–167, 1905.

(*19*) Colonial Bacteriological Institute: Report of the Director (A. Edington) for the year 1896.

(*20*) Watkins-Pitchford, W.: The prevalence of cancer amongst the native races of Natal and Zululand during the four years 1906–1909. M. J. South Africa 20: 257–260, 1925.

(*21*) ———: Report of the Natal Cancer Research Committee for the year 1906. Natal Govt. Gaz. 23rd July: 784–786, 1907.

(*22*) ———: Report of the Natal Cancer Research Committee for the years 1907–1909. Pietermaritzburg, Times Printing & Publishing Co., Ltd., 1910, 14 pp.

(23) CASALIS, G. A.: The incidence of cancer in South Africa. Transvaal M. J. 6: 119–124, 1911.
(24) VELLACOTT, P. N.: A note on malignant disease in the O. R. C. South African M. Rec. 8: 51–54, 1910.
(25) Report of the Medical Officer of Health, 1910, covering the period 1st July, 1906–30th June, 1909. Johannesburg.
(26) Report of the Medical Officer of Health, 1912, covering the period 1st July, 1909–30th June, 1911. Johannesburg.
(27) Report of the Medical Officer of Health, 1913, covering the period 1st July, 1912–30th June, 1913. Johannesburg.
(28) Report of the Medical Officer of Health, 1916, covering the period 1st July, 1914–30th June, 1915. Johannesburg.
(29) Report of the Medical Officer of Health, 1921, covering the period July, 1919–June, 1920. Johannesburg.
(30) Report of the Medical Officer of Health, 1917, covering the period 1st July, 1915–30th June, 1916. Johannesburg.
(31) Annotation: Transvaal M. J. 8: 162–163, 1913.
(32) Report of the Medical Officer of Health, 1925, covering the period 1922–1923, 1923–1924. Johannesburg.
(33) SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH: Annual report for the year ended 31st December, 1920. P. 34.
(34) PIRIE, J. H. H.: Hepatic carcinoma in natives and its frequent association with schistosomiasis. M. J. South Africa 17: 87–97, 1921.
(35) TURNER, G. A., and BREBNER, I. W.: The serum treatment of cerebrospinal meningitis. M. J. South Africa 10: 205–225, 1915.
(36) SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH: Annual report for the year ended 31st December, 1922.
(37) HAWARDEN, S. A.: Report of a case of primary liver cancer. Proc. Transvaal Mine M. Offrs' Assoc. 1: (No. 12) 1, 1922.
(38) MCGREGOR, A. L.: Report of case. Proc. Transvaal Mine M. Offrs' Assoc. 3: (No. 3) 4, 1923.
(39) GOLDSMITH, A. W.: Report of case. Proc. Transvaal Mine M. Offrs' Assoc. 3: (No. 5) 4, 1924 and 3: (No. 7) 9, 1924.
(40) MILLER, L. E.: Report of 2 cases. Proc. Transvaal Mine M. Offrs' Assoc. 3: (No. 10) 12, 1924.
(41) LEIPOLDT, C. L.: A note on cancer among natives. M. J. South Africa 19: 206–207, 1924.
(42) MACFARLANE: Personal communication to Dr. Brock, and so to Leipoldt.
(43) MACVICAR, N.: Notes on cancer among native people. South African M. Rec. 23: 315–316, 1925.
(44) DES LIGNERIS, M. J. A.: Tumours in Northern Transvaal. An analysis of twenty years statistics in a country hospital. J. M. A. South Africa 25: 102–107, 1927.
(45) KEEN, P.: Malignancy in the Bantu. Leech, Johannesburg, 22: 12–13, 1952.
(46) BEYERS, C. F.: Incidence of surgical diseases among the Bantu races of South Africa. J. M. A. South Africa 1: 606–612, 1927.
(47) Editorial: J. M. A. South Africa 25: 605–606, 1927.
(48) ORENSTEIN, A. J.: *Quoted by* Beyers, C. M., J. M. A. South Africa 25: 606–609, 1927.
(49) FISCHER, W. O.: A preliminary report on 1,402 consecutive autopsies on native mine workers. J. M. A. South Africa 3: 511–516, 1929.
(50) ———: Record of 561 consecutive autopsies on native mine workers. South African M. J. 6: 359–361, 1932.
(51) ———: Primary carcinoma of the liver in East African natives. Is it caused by an infective agent? South African M. J. 24: 869–871, 1950.
(52) Mozambique convention regulating the introduction of native labour from Mozambique into the Province of the Transvaal, railway matters, and the

commercial intercourse between the Union of South Africa and the Colony of Mozambique, entered into between the Government of the Union of South Africa and the Government of the Portuguese Republic at Pretoria on the 11th September, 1928. Pretoria, Government Printer, 8063–14/9/28.

(*53*) HIGGINSON, J. H., and DE MEILLON, B.: Hepatic fibrosis and cirrhosis in relation to mild *Schistosoma haematobium* infestation in man. Arch. Path. 60: 341–346, 1955.

(*54*) STRACHAN, A. S.: Observations on the incidence of malignant disease in South African natives. J. Path. & Bact. 39: 209–211, 1934.

(*55*) BERMAN, C.: The clinical features of primary carcinoma of the liver in the Bantu races of South Africa. South African J. M. Sc. 5: 92–109, 1940.

(*56*) ORENSTEIN, A. J.: Report of the Central Mining-Rand Mines Group Health Department, 1936. P. 54.

(*57*) FREEDMAN, J.: Personal communication, 1955.

(*58*) DES LIGNERIS, M. J. A.: Cancer in South African natives. Communications of the 2nd International Congress of Scientific and Social Campaign Against Cancer, 1936, pp. 602–611.

(*59*) PEARSON, R. C. T.: Valedictory address. Proc. Transvaal Mine M. Offrs' Assoc. 33: 111–118, 1954.

(*60*) MARTINY, O.: Personal communication, 1955.

(*61*) CLEMMESEN, J., and NIELSEN, A.: The incidence of malignant diseases in Denmark 1943 to 1947. Acta Unio internat. contra cancrum 8: 140–149, 1952.

(*62*) SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH: Annual report for the years 1948, p. 33; 1949, p. 43; 1950, p. 65; 1953, p. 79.

(*63*) ROSSET, J. A.: Personal communication, 1955.

(*64*) NYSTROM: Kräftsjukdomarna i Sverige, Stockholm. 1922. *Quoted by* Dahlberg, G.: Statistical Methods for Medical and Biological Students. London, G. Allen & Unwin, Ltd., 1940, pp. 88, 89.

(*65*) HIGGINSON, J.: A rational approach to cancer surveys in the Bantu. Leech, Johannesburg, 22: 27–30, 1952.

(*66*) BERMAN, C.: Primary carcinoma of the liver in the Bantu races of South Africa. South African J. M. Sc. 5: 54–72, 1940.

(*67*) ———.: The clinical features of primary carcinoma of the liver. Clin. Proc., Cape Town, 3: 323–330, 1944.

(*68*) PRATES, M.: Primary malignant tumours of the liver in Mozambique natives. South African M. J. 14: 95–99, 1940. Also printed in the report: Primeiro Congresso Medico de Lourenço Marques II: 71–79, 1938. Lourenço Marques, Imprensa Nacional de Mozambique, 1940.

(*69*) ———: A bilharziose na Africa oriental Portuguesa e a sua importancia na etiologia dos carcinomas primitivos do figado dos indigenas. An. Inst. med. trop. 5: 149–174, 1948.

(*70*) STIBBE, E. P.: A preliminary note on the relationship of weight to height, age, and work in different native races. Proc. Transvaal Mine M. Offrs' Assoc. 3: (No. 11) 9–14, 1924.

(*71*) DORN, H. F., and CUTLER, F. J.: Morbidity from Cancer in the United States. Pt. I. Variation in incidence by age, sex, race, marital status and geographic region. Washington, D. C., U. S. Government Printing Office, Pub. Health Mono. No. 29, 1955, 121 pp.

(*72*) FINDLAY, G. M.: Observations on primary liver carcinoma in West African soldiers. J. R. micr. Soc. 70: 166–172, 1950.

(*73*) HIGGINSON, J.: Malignant neoplastic disease in the South African Bantu. Cancer 4: 1224–1231, 1951.

(*74*) OETTLÉ, A. G.: Racial differences in cancer in South Africa. South African Biol. Soc. Pamphlet No. 17: 1–10, 1955.

(*75*) GARIN, H.: Personal communication, 1955.

(*76*) HIGGINSON, J., and OETTLÉ, A. G.: Carcinoma of the Oesophagus in the South African Bantu. Acta Unio internat. contra cancrum. In press.

(77) PELLER, S.: Lung cancer among mine workers in Joachimsthal. Human Biol. 11: 130–143, 1939.

(78) LONSDALE, K.: The scientist's responsibilities as a citizen. *In* Biological Hazards of Atomic Energy (Haddow, A., ed.). Oxford, Clarendon Press, 1952, pp. 211–225.

(79) MINERS' PHTHISIS MEDICAL BUREAU: Report upon the work for the three years ended 31st July, 1932. Pretoria, Government Printer, 1933.

(80) PIROW, H.: Distribution of the pebbles in the Rand banket etc. Tr. geol. Soc. South Africa 23: 97, 1920.

(81) BERMAN, C.: Etiology of primary carcinoma of the liver with special reference to the Bantu races of South Africa. South African J. M. Sc. 6: 145–156, 1941.

(82) ERÄNKÖ, O.: Quantitative Methods in Histology and Microscopic Histochemistry. Basel, S. Karger, 1955.

# The Incidence of Primary Carcinoma of the Liver in the Southern Bantu. II. Preliminary Report on Incidence in Johannesburg [1, 2]

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At the beginning of 1953 a cancer registry was set up at this Institute to ascertain the incidence of cancer in an urban Bantu population. This survey has not yet been completed, but the preliminary figures for primary carcinoma of the liver during the first 2 years are presented to complement the first part of the study (*1*). These figures clearly demonstrate that the incidence in urban Bantu is much higher than in the population of Denmark (*2*) and the United States of America [Dorn and Cutler (*3*)].

## Methods

Although all malignant tumors diagnosed in the Johannesburg magisterial district are registered, these preliminary calculations have been limited to certain urban and peri-urban townships, as adequate demographic data are not yet available for the entire area. Cancer registrations were carefully screened and only residents of these selected townships were considered—miners and visitors from elsewhere being excluded. In our final report we intend to survey the entire Bantu population of Johannesburg, to avoid the possible error caused by undetected internal migration, although the effect of such migration is unlikely to be large.

### *Collection of Cases*

The relative centralization of free medical services for the Bantu and the almost complete absence of private facilities for surgery or radiotherapy of cancer have made it easier to obtain records of cases.

Cases were registered from hospitals (by their medical attendants) and from certificates of death. To ensure completeness, we also examined

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[3] The social survey of Alexandra Township was directed by Miss H. Navid of the Entokosweni Social Centre there, and we are indebted to her and her staff for their collaboration.

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hospital case records for this area, radiotherapy records, and biopsy and necropsy records. It was found that relatively few cases were treated by private practitioners, who were requested to register cases in their care.

To avoid errors regarding the domicile, patients were questioned in the hospital or at home by social workers. Bantu personnel were found most suitable for this purpose, being unconnected with the hospital and able to speak one or more Bantu languages.

### *Definition of the Population-at-Risk*

Wartime and postwar development have led to rapid changes in the Bantu population. For example, in 1936 Moroka, Jabavu, and Kliptown, and the major portion of Orlando township did not exist, and Alexandra township possessed 15,945 inhabitants. In 1953, the new townships housed an estimated 148,000 and Alexandra township 76,000.

The latest census (1951) provides figures for total population, but the age and sex distribution are unavailable, and we do not know to what extent those derived from the 1946 census would be applicable to the present population. However, as certain restricted surveys are available, giving age and sex distribution for individual townships (table 1) in Johannesburg, this report has been limited to these and to others of similar type to which the figures for comparable townships might arbitrarily be applied. The differences between townships (table 1) are relatively slight, and errors caused in this approximation are not likely to affect the results seriously.

The sources of information and approximations involved are given in table 2, while the totals in each age and sex group at risk are given in table 3.

TABLE 1.—*Age-sex distribution of certain Bantu population groups in Johannesburg*

| Age group | Sophiatown and Martindale | | Orlando East | | Orlando West | | Jabavu | | Alexandra | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F |
| 0–4 | 13. 2 | 16. 9 | 16. 00 | 15. 58 | 16. 9 | 17. 3 | 20. 7 | 20. 3 | 15 | 17 |
| 5–14 | 15. 0 | 21. 6 | 20. 92 | 22. 23 | 22. 9 | 24. 7 | 23. 8 | 26. 0 | 17 | 22 |
| 15–24 | 15. 5 | 15. 2 | 18. 07 | 18. 30 | 17. 1 | 18. 0 | 11. 3 | 14. 9 | 13 | 19 |
| 25–34 | 21. 3 | 22. 6 | 12. 38 | 13. 72 | 12. 5 | 15. 3 | 14. 4 | 22. 1 | 22 | 19 |
| 35–44 | 19. 7 | 12. 2 | 13. 34 | 14. 42 | 14. 3 | 11. 9 | 19. 6 | 10. 2 | 20 | 12 |
| 45–54 | 8. 7 | 6. 9 | 10. 04 | 6. 87 | 8. 4 | 5. 2 | 7. 6 | 3. 4 | 10 | 6 |
| 55–64 | 4. 5 | 3. 3 | 4. 45 | 3. 93 | 2. 8 | 2. 9 | 1. 8 | 2. 0 | 2 | 3 |
| 65–74 | 1. 3 | 1. 4 | 1. 83 | 1. 56 | 1. 1 | 1. 2 | 0. 5 | 0. 9 | 1. 3 | 1 |
| 75+ | 0. 8 | 0 | 0. 46 | 1. 26 | 0. 5 | 1. 0 | 0. 1 | 0. 3 | 0. 5 | 0. 5 |
| Unknown | — | — | 2. 50 | 2. 13 | 3. 5 | 2. 5 | | | | |
| Total | 100. 0 | 100. 1 | 99. 99 | 100. 00 | 100. 0 | 100. 0 | 100. 0 | 100. 0 | 100. 8 | 99. 5 |

### *Main Characteristics of the Population-at-Risk*

It will be noticed that older age groups are poorly represented, and a sharp drop occurs in the region of the forties, the drop occurring rather earlier in females than in males. Because of the small number of aged

people, marked variability can therefore be expected in calculated rates for the older groups. The figures emphasize the virtual impossibility of interpreting crude cancer ratios derived from a population with such irregular age distribution.

TABLE 2.—*Estimated total populations of the selected townships used in establishing cancer rates*

| Township | Estimated population | Origin of estimate* | Source of age and sex distribution* |
|---|---|---|---|
| Alexandra | 74, 822 | (a) | (b) |
| Orlando East | 31, 756 | (c) | (d) |
| Orlando West | 18, 814 | (c) | (d) |
| Orlando Shelters | 18, 330 | (e) | (b) |
| Albertynsville and Kliptown | 9, 123 | (f) | (b) |
| Malay township | 2, 549 | (f) | (h) |
| Pimville | 17, 400 | (g) | (b) |
| Jabavu | 27, 200 | (g) | (i) |
| Moroka | 43, 000 | (g) | (i) |
| Sophiatown, Martindale | 39, 186 | (c) | (c) |
| Newclare | 14, 561 | (c) | (c) |
| Western native township | 13, 240 | (f) | (h) |
| Eastern native township | 4, 171 | (f) | (i) |
| Total | 314, 152 | | |

*(a) Our survey of Alexandra township, 1953, gave a total of 77,112. The figure of 2,290 non-Bantu was obtained from the 1951 census and subtracted.
(b) Our survey, 1953, of Alexandra township.
(c) Feldman's unpublished survey, 1950 (*4*).
(d) " " " , 1952 (*5*).
(e) Census estimate, 1953, less Feldman's figures for Orlando, East and West.
(f) Census, 1951.
(g) Census, 1951, with correction for estimated population, 1953.
(h) Adopting the figures for Sophiatown and Martindale.
(i) Feldman's unpublished sample study, 1952, of this township.

TABLE 3.—*Estimated total population of Bantu in certain selected Johannesburg urban and peri-urban townships*

| Age group | Males | | Females | |
|---|---|---|---|---|
| | Number | Percentage | Number | Percentage |
| 0–4 | 24, 227 | 15. 35 | 26, 828 | 17. 12 |
| 5–14 | 28, 275 | 17. 85 | 35, 424 | 22. 6 |
| 15–24 | 22, 414 | 14. 5 | 27, 593 | 17. 64 |
| 25–34 | 31, 221 | 19. 8 | 30, 505 | 19. 5 |
| 35–44 | 29, 801 | 18. 9 | 19, 319 | 12. 7 |
| 45–54 | 14, 902 | 9. 43 | 9, 605 | 6. 14 |
| 55–64 | 4, 688 | 2. 96 | 4, 678 | 2. 99 |
| 65–74 | 1, 944 | 1. 23 | 1, 827 | 1. 17 |
| 75+ | 850 | 0. 54 | 764 | 0. 49 |
| Total | 158, 331 | | 156, 543 | |

### *Difference between Total Population and Population-at-Risk*

In assessing the accuracy of rates it is necessary to know what proportion of patients escapes detection. This was especially necessary in view

of the widely held belief that primitive groups would be especially unwilling to seek modern Western medical assistance. In this survey, however, the contrary was found. For example, in Alexandra township the great majority of those interviewed, was prepared to seek the advice of Western medical practitioners when ill. About 1 percent stated that they would not do this, in many cases on account of religious teaching. Since, however, a death certificate is necessary for burial, even those with religious grounds for avoiding medical assistance consult a doctor when a patient is *in extremis* to avoid the necessity of a medico-legal necropsy before issue of a burial order.

An unknown percentage leaves the city to convalesce in the country or because of inability to continue earning, but a survey of attitudes suggests that this group is small.

Despite these losses, therefore, we feel justified in regarding the population-at-risk as essentially synonymous with the total population.

### *Period of Study*

The study covered a period of 2 calendar years—January 1, 1953, to December 31, 1954. Cases diagnosed in a hospital were registered according to the date of first admission when cancer was diagnosed. For those never admitted to a hospital, the date of registration or of the death certificate was taken, whichever was earlier.

## Results and Discussion

The age- and sex-specific rates for all cancers calculated from this series of approximations have given reasonably regular curves, corresponding fairly closely with those reported for the American Negro. The curves for certain specific cancers, such as carcinoma of the cervix, also coincide with those reported for American Negroes. Furthermore, our 1953 and 1954 figures for primary carcinoma of the liver were essentially of the same order.

The number of cases of primary liver cancer, classified according to stringency of diagnosis, is given in table 4. As clinical diagnoses are fallible, the total number may well be somewhat overestimated. This is especially likely to affect the older age groups where the proportion of cases diagnosed clinically was higher (table 4). It is possible that the accuracy of clinical diagnosis has been improved by the use of batteries of hepatic function tests—these are more readily available for hospital cases.

In table 5 are shown the rates derived from the total Bantu registrations and those from Danish and U. S. populations. It is evident that the Bantu rates, calculated from 2 or 3 cases in an age group, cannot be precise, but the trends have a greater significance than individual figures.

The most satisfactory method of camparison, however, is to contrast the observed number of cancers in the Bantu population with the expected number for other populations (table 6) of the same size, age, and sex

TABLE 4.—*Liver-cancer cases with source of diagnosis from the population of table 3*

| Age group | Males | | | | Females | | |
|---|---|---|---|---|---|---|---|
| | Total cases 1953–1954 | Histologically confirmed | Others diagnosed in hospital | Diagnosed by general practitioners | Total cases | Histologically confirmed | Diagnosed by general practitioners |
| 5–14 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 15–24 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| 25–34 | 10 | 5 | 4 | 1 | 1 | 1 | 0 |
| 35–44 | 21 | 14 | 5 | 2 | 2 | 1 | 1 |
| 45–54 | 9 | 4 | 1 | 4 | 3 | 1 | 2 |
| 55–64 | 9 | 4 | 0 | 5 | 7 | 3 | 4 |
| 65–74 | 6 | 1 | 1 | 4 | 3 | 0 | 3 |
| 75+ | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| Total | 57 | 30 | 11 | 16 | 19 | 7 | 12 |

TABLE 5.—*Total liver cancer in different races per 100,000 per annum*

| Age group | Bantu | Danes | U. S. Whites | U. S. Negroes |
|---|---|---|---|---|
| **Males** | | | | |
| 5–14 | 1. 8 | 0 | 0. 1 | 0 |
| 15–24 | 2. 2 | 0 | 0. 2 | 0 |
| 25–34 | 16 | 0 | 0. 3 | 1. 2 |
| 35–44 | 37 | 0 | 0. 9 | 3. 1 |
| 45–54 | 30. 2 | 0. 5 | 4. 4 | 12. 8 |
| 55–64 | 96 | 1. 5 | 21. 9 | 14. 7 |
| 65–74 | 154 | 4. 5 | 25. 0 | 17. 0 |
| 75+ | 0 | 6. 5 | 40. 5 | 27. 1 |
| **Females** | | | | |
| 5–14 | 0 | 0 | 0. 1 | 0 |
| 15–24 | 1. 8 | 0 | 0. 1 | 0. 7 |
| 25–34 | 1. 6 | 0 | 0. 1 | 1. 0 |
| 35–44 | 5. 8 | 0 | 0. 6 | 1. 7 |
| 45–54 | 15. 6 | 1. 0 | 4. 6 | 4. 0 |
| 55–64 | 76. 1 | 0. 5 | 13. 4 | 9. 5 |
| 65–74 | 82 | 1. 5 | 21. 6 | 6. 6 |
| 75+ | 131 | 4. 5 | 47. 7 | 15. 7 |

distribution. This suggests that at Bantu rates the number of hepatomas encountered is about 50 to 100 times higher than what would be expected at Danish rates and 5 to 10 times higher than at U. S. rates. Since the age of onset differs, as well as the slope of the curve, the ratios of observed to expected numbers will depend on the nature of the standard population chosen—here the Bantu population has been the standard.

The rates indicate, furthermore, that incidence rises with age. This fact has been missed by earlier students of cancer ratios. Berman (*6*), for example, held that, in the Bantu, carcinoma of the liver is mainly a disease of young adults. If allowance is made for the number represented in each age group within the population, the increase with age is apparent and follows the pattern of most carcinomas. What is striking, however, is the early age of onset—a fact that has been noted by most observers.

This early incidence is responsible for the reported variation in apparent frequency of this cancer. In the male population under 45, about 50 percent of all carcinomas arose in the liver; above this age the proportion declined rapidly.

TABLE 6.—*Number of cases of liver cancer found compared with expected number from a population of identical size and age and sex groupings over a 2-year period*

| Age group | Population at risk | No. of cases | Expected number in similar population | | |
|---|---|---|---|---|---|
| | | | Denmark | U. S. whites | U. S. Negroes |
| | | | **Males** | | |
| 5–14 | 28, 275 | 1 | 0 | .06 | 0 |
| 15–24 | 22, 414 | 1 | 0 | .09 | 0 |
| 25–34 | 31, 221 | 10 | 0 | .19 | .37 |
| 35–44 | 29, 801 | 21 | 0 | .56 | 1.85 |
| 45–54 | 14, 902 | 9 | .15 | 1.31 | 3.81 |
| 55–64 | 4, 688 | 9 | .14 | 2.05 | 1.38 |
| 65–74 | 1, 944 | 6 | .18 | .97 | .68 |
| 75+ | 850 | 0 | .11 | .68 | .46 |
| Total | 158, 331 | 57 | .58 | 5.91 | 8.55 |
| Factor | | 1.0 | 99 | 9.7 | 7.45 |
| | | | **Females** | | |
| 5–14 | 35, 424 | 0 | 0 | .07 | 0 |
| 15–24 | 27, 593 | 1 | 0 | .06 | .36 |
| 25–34 | 30, 505 | 1 | 0 | .06 | .60 |
| 35–44 | 19, 319 | 2 | 0 | .21 | .66 |
| 45–54 | 9, 605 | 3 | .20 | .87 | .77 |
| 55–64 | 4, 678 | 7 | .05 | 1.25 | .88 |
| 65–74 | 1, 827 | 3 | .06 | .79 | .24 |
| 75+ | 764 | 2 | .07 | .73 | .24 |
| Total | 156, 543 | 19 | .38 | 4.04 | 3.75 |
| Factor | | 1.0 | 50 | 4.7 | 5.1 |

The greater susceptibility of males to liver cancer is confirmed, but the sex ratio appears to vary with age, as demonstrated earlier (*1*). Female cases are relatively rarer in the younger age groups, but become more common in the older groups, when the disparity between the sexes tends

TABLE 7.—*Ratio of male to female rates, according to diagnostic stringency*

| Age group | Histologic | | All hospital cases (histologic and clinical) | | Total | |
|---|---|---|---|---|---|---|
| | Rates | Ratio | Rates | Ratio | Rates | Ratio |
| 0–14 | 1.8 / 0.0 | | 1.8 / 0.0 | | 1.8 / 0.0 | |
| 15–24 | 2.2 / 1.8 | 1.2 | 2.2 / 1.8 | 1.2 | 2.2 / 1.8 | 1.2 |
| 25–34 | 8.0 / 1.6 | 5.6 | 14.4 / 1.6 | 9.0 | 16.0 / 1.6 | 10.0 |
| 35–44 | 23.4 / 2.6 | 9.2 | 31.8 / 2.6 | 12.2 | 37.0 / 5.8 | 7.1 |
| 45–54 | 13.4 / 5.4 | 2.4 | 16.8 / 5.4 | 3.1 | 30.2 /15.6 | 2.0 |
| 55–64 | 42.8 /32.0 | 1.3 | 42.8 /32.0 | 1.3 | 96.0 /76.1 | 1.3 |
| 65–74 | 25.7 / 0.0 | | 51.4 / 0.0 | | 154.0 /82.0 | 1.9 |

to disappear (table 7). In this survey, below the age of 45 the male incidence is approximately 6 times that in females. Above 45 the disease is about twice as common in males. This applies to total as well as to histologically confirmed cases.

Evidently crude sex ratios for carcinoma of the liver are dependent on so many variables that they should be replaced, where possible, by age-specific sex ratios.

## Summary

1) Preliminary age- and sex-specific liver-cancer rates are presented for the Bantu population of certain townships in the Johannesburg district of the Union of South Africa. The possibility of extracting cancer rates from relatively limited data is demonstrated.

2) The rates are 50 to 100 times higher than those expected from a Danish population and 5 to 10 times higher than those expected from United States populations of age and sex distribution similar to this Bantu group.

3) The earlier incidence of hepatoma in the Bantu is confirmed, but the disease is not confined to the younger age groups and its incidence increases steadily with age.

4) The incidence is higher in males than in females, but the sex ratio varies with age, being very much higher in the first 5 decades than thereafter.

## References

(*1*) OETTLÉ, A. G.: The incidence of primary carcinoma of the liver in the southern Bantu. I. Critical review of the literature. J. Nat. Cancer Inst. 17: 000–000, 1956.

(*2*) CLEMMESEN, J., and NIELSEN, A.: The incidence of malignant disease in Denmark 1943 to 1947. Acta Unio. internat. contra cancrum 8: 140–159, 1952.

(*3*) DORN, H. F., and CUTLER, S. J.: Morbidity from cancer in the United States. Pt. I. Variation in incidence by age, sex, race, marital status and geographic region. Washington, D. C., U. S. Government Printing Office, Pub. Health Mono. No. 29, 1955, 121 pp.

(*4*) FELDMAN, G. R.: Personal communication.

(*5*) ———: Personal communication.

(*6*) BERMAN, C.: Primary Carcinoma of the Liver. London, H. K. Lewis, 1951, 164 pp.

# On the Heredity of Glioblastoma [1,2]

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During the past few decades various reports have appeared on the significance of inherited factors in the development of malignant tumors (*1–4*). On the basis of the literature available Kemp (*5*) in 1948 concluded that the influence of heredity consisted partly of a general predisposition to develop malignant growths and partly of factors governing the localization of tumors. Koch (*6,7*) has claimed that inherited factors also play an etiologic role in the development of intracranial tumors, especially glioblastomas. One may object, however, that his studies are based on case reports only.

This paper reports a systematic proband study of patients with glioblastomas.

## Materials and Methods

The probands were patients with microscopically verified glioblastomas (angionecrotic or cellular) obtained from the Neurosurgical Department of the Copenhagen University Clinic from 1945 to 1953. The total series comprised 189 patients. From this material we excluded altogether 20 deceased patients about whom family data could not be procured. In 10 of these cases it was impossible to identify the patient because false or insufficient data had been given to the hospital; 1 patient was an adopted child; 4 patients had no surviving relatives who could supply information and in 5 cases the surviving families refused to collaborate. So the final series studied comprised 169 families.

Weinberg's proband method with minor modifications was used. The following groups of relatives were examined: parents, siblings, children, parents' siblings, and half siblings. Parents' siblings were included only in families where at least 1 sibling was living and able to supply data. Relatives who had emigrated were not included.

The control series was derived from the major control material available at the University Institute for Human Genetics, consisting of persons

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[3] With the technical assistance of H. Nørgård and G. Gotfredsen.

from different social groups: craftsmen, clerks, nurses, old-age pensioners, and others. In this control series no proband was included with a verified or suspected diagnosis of cancer. The controls for this study were selected by chance from the major material in the following way: Each patient of the proband series was paired with the first person in the control series who was of the same sex and the same age as the proband, the maximum deviation being $\pm$ 1 year. In the control group, as well as in the proband series, the relatives were paired in the same manner.

Within the groups of relatives examined, information was obtained on all cases of intracranial tumor and cancer and, for deceased persons, on the causes of death—in most instances through death certificates or hospital records.

## Results

Tables 1 and 2 present the number of cases of intracranial tumor and of cancer among relatives of controls and probands, according to age groups. It may be seen from these tables that the control series is very similar to the proband series in the distribution of relatives among the different groups. Hence, a direct comparison may be justified. Furthermore, there is no significant difference in the sex ratio in the 2 series.

A total of 5 cases of intracranial tumor occurred among the 1,744 relatives in the proband series and 5 cases also among the 1,625 relatives in the control series.

The data regarding secondary cases in the proband series are relatively inhomogeneous. Only 1 of the patients had received neurosurgical treatment, and this is the only case with microscopic verification (astrocytoma at the fissure of Sylvius). In 2 of the remaining cases the tumors were *post-mortem* findings, and in 2 the diagnosis was based merely on clinical findings.

In the 5 secondary cases of the control series, the diagnoses were based on microscopic examination of the tumors (oligodendroglioma and glioblastoma) in 2 patients, autopsy findings in 1 patient, and clinical findings in 2 patients.

At the time of examination, 134 of the proband series and 130 of the control series had died from cancer.

Table 3 gives the calculation of risk of dying from cancer for close and distant relatives in both the proband and control series. This calculation is based on the life-table method and was carried out along the lines indicated by Nyholm and Helweg-Larsen (*8*). These data are interpreted as the risk of dying from cancer which the relatives undergo if they reach a given age without dying of other causes. For each age group the number of cancer deaths among relatives and the number examined and still surviving at the time of the investigation are computed. A person is counted as half a unit in whatever age group his death or our examination took place. The calculation of the risk of dying from cancer previous to some age limit $x$, denoted $R_x$ (here computed for the age of 80, $R_{80}$), is based

TABLE 1.—*Incidence of cancer and intracranial tumor among relatives of probands**

| Age group | Fathers | | Mothers | | Brothers | | Sisters | | Sons | | Daughters | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead |
| 10–19 | | | | | | 9 | | 9 | 25 | 2 | 18 | |
| 20–29 | | | | | 1 | 13 | 2 | 7 | 41 | 1 | 42 | 2 |
| 30–39 | | 5 (1) | | 4 (1) | 15 | 12 (*1*) | 16 | 8 (1) | 50 | 2 | 39 | 3 (*1*) |
| 40–49 | | 6 (2) | | 13 (4+*1*) | 64 | 8 (1) | 65 (1) | 12 (9) | 19 (1) | | 20 | 2 (1) |
| 50–59 | 2 | 23 (3) | 2 | 21 (11) | 92 (1) | 19 (7) | 99 (3) | 17 (6+*1*) | 2 | 1 | | |
| 60–69 | 5 | 31 (4) | 5 | 21 (5) | 50 (1) | 12 (2) | 59 | 13 (4) | | | | |
| 70–79 | 17 | 46 (10) | 20 (2) | 45 (8+*1*) | 16 (1) | 4 | 27 (1) | 7 (2) | | | | |
| 80–89 | 5 (2) | 24 | 11 | 25 (3) | 1 | | 2 | 1 | | | | |
| 90–99 | 1 | 2 | | 2 (1) | | | | | | | | |
| Total | 30 (2) | 137 (20) | 38 (2) | 131 (33+*2*) | 239 (3) | 77 (10+*1*) | 270 (5) | 74 (22+*1*) | 137 (1) | 6 | 119 | 7 (1+*1*) |

| Age group | Paternal uncles | | Paternal aunts | | Maternal uncles | | Maternal aunts | | Half brothers | | Half sisters | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead |
| 10–19 | | 3 | | 4 | | 3 | | 7 | | | | |
| 20–29 | | 3 | | 2 | | 6 | | 2 | 1 | | | |
| 30–39 | | 1 | | 3 | | 3 (1) | | 4 (2) | 3 | | 1 | |
| 40–49 | | 4 (1) | 3 | 5 | | 8 (1) | 1 | 5 (2) | 5 | | 3 | |
| 50–59 | 9 | 6 (2) | 5 | 6 (3) | 5 | 12 (2) | 6 | 7 (2) | 3 | 2 | 7 | |
| 60–69 | 14 | 12 (3) | 21 | 8 (2) | 14 | 12 (3) | 20 | 9 (4) | 3 | | 3 | |
| 70–79 | 21 | 14 (3) | 27 | 13 (4) | 25 | 11 (2) | 21 (2) | 16 (3) | 1 | | 1 | |
| 80–89 | 3 | 9 | 8 | 5 (1) | 16 | 7 (1) | 8 | 6 (1) | | | | |
| 90–99 | 1 | | 1 | | | | 1 | | | | | |
| Total | 48 | 52 (9) | 65 | 46 (10) | 60 | 62 (10) | 57 (2) | 56 (14) | 16 | 2 | 15 | |

*First figure in parentheses=number with cancer; italicized figure=number with intracranial tumor.

TABLE 2.—*Incidence of cancer and intracranial tumor among relatives of controls**

| Age group | Fathers | | Mothers | | Brothers | | Sisters | | Sons | | Daughters | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead |
| 10–19 | | | | | 3 | 5 | | 9 | 11 | 4 *(1)* | 13 | 2 *(1)* |
| 20–29 | | | | 4 | 8 | 9 | 8 | 9 | 41 (1) | 1 | 29 | |
| 30–39 | | 9 | | 11 | 20 | 10 | 14 | 2 | 45 | 2 | 28 | 3 (1) |
| 40–49 | | 13 (3) | 4 | 7 (3) | 42 | 16 (1+*1*) | 35 | 14 (3) | 22 | | 24 | 1 (1) |
| 50–59 | 9 | 26 (5) | 5 | 8 (1) | 61 | 18 (4+*1*) | 66 (1) | 13 (9) | 2 | | 4 | |
| 60–69 | 8 | 28 (7) | 11 | 21 (4) | 47 (1) | 26 (5) | 70 | 20 (5) | 3 | | | |
| 70–79 | 5 | 40 (8) | 12 | 40 (9) | 24 | 13 (5) | 20 | 16 (3) | | | | |
| 80–89 | 9 | 21 (4) | 7 | 30 (2) | 6 | 1 | 4 | 1 | | | | |
| 90–99 | | 2 | | 10 | | | | 1 | | | | |
| Total | 31 | 139 (27) | 39 | 131 (19) | 211 (1) | 98 (15+*2*) | 217 (1) | 85 (20) | 124 (1) | 7 *(1)* | 98 | 6 (2+*1*) |

| Age group | Paternal uncles | | Paternal aunts | | Maternal uncles | | Maternal aunts | | Half brothers | | Half sisters | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead | Alive | Dead |
| 10–19 | | 3 | | 1 | | 3 | | 2 | | 1 | | |
| 20–29 | | 3 | | 1 | | 4 | | 1 | | | | 1 |
| 30–39 | | 1 | 1 | 2 | 1 | 4 | | 6 | 2 | | | |
| 40–49 | 2 | 4 (1) | 5 | 4 | 8 | 13 (3) | 3 | 4 | 2 | 3 (1) | | |
| 50–59 | 10 | 5 | 11 | 6 (2) | 9 | 7 (1+*1*) | 9 | 6 (3) | 6 | 2 | 2 | |
| 60–69 | 14 | 14 (3) | 13 | 7 (3) | 12 | 12 (2) | 15 (1) | 10 (2) | 6 | 2 | 1 | |
| 70–79 | 11 | 8 | 10 | 12 (4) | 22 | 16 (7) | 18 | 10 (3) | 3 | | 1 | |
| 80–89 | 7 | 4 (1) | 4 | 7 (2) | 7 | 9 (1) | 8 | 7 (2) | 1 | 1 (1) | | |
| 90–99 | 1 | | 1 | | | 2 | 2 | 3 | | | | |
| Total | 45 | 42 (5) | 45 | 40 (11) | 59 | 70 (14+*1*) | 55 (1) | 49 (10) | 20 | 9 (2) | 4 | 1 |

*First figure in parentheses=number with cancer; italicized figure=number with intracranial tumor.

TABLE 3.—*Risk of dying from cancer for proband and control series**†*

| Age group | Close relatives | | | | | | Distant relatives | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Proband series | | | Control series | | | Proband series | | | Control series | | |
| | A | L | A/L | A | L | A/L | A | L | A/L | A | L | A/L |
| 10–19 | 0 | 1234 | — | 2 | 1163 | 0. 0017 | 0 | 471 | — | 0 | 434 | — |
| 20–29 | 0 | 1148 | — | 0 | 1085 | — | 0 | 455 | — | 0 | 424 | — |
| 30–39 | 5 | 1016 | 0. 0049 | 1 | 958 | 0. 0010 | 3 | 441 | 0. 0068 | 0 | 411 | — |
| 40–49 | 18 | 835 | 0. 0216 | 11 | 797 | 0. 0138 | 4 | 416 | 0. 0096 | 5 | 378 | 0. 0132 |
| 50–59 | 28 | 591 | 0. 0474 | 17 | 602 | 0. 0282 | 9 | 365 | 0. 0247 | 7 | 318 | 0. 0220 |
| 60–69 | 15 | 354 | 0. 0424 | 25 | 379 | 0. 0660 | 12 | 273 | 0. 0440 | 10 | 228 | 0. 0439 |
| 70–79 | 21 | 165 | 0. 1272 | 25 | 177 | 0. 1412 | 12 | 140 | 0. 0857 | 14 | 120 | 0. 1172 |
| 80–89 | 3 | 40 | — | 6 | 53 | — | 3 | 34 | — | 7 | 37 | — |
| | $\Sigma(A/L)=0.2435$<br>$R_{80}=21.62\%$<br>Var. $R_{80}=6.15$ | | | $\Sigma(A/L)=0.2519$<br>$R_{80}=22.27\%$<br>Var. $R_{80}=6.28$ | | | $\Sigma(A/L)=0.1708$<br>$R_{80}=15.70\%$<br>Var. $R_{80}=6.25$ | | | $\Sigma(A/L)=0.1963$<br>$R_{80}=17.81\%$<br>Var. $R_{80}=8.63$ | | |

*A: Number of persons who died from cancer in the age interval indicated.
†L: Number of persons who at the time of investigation had passed the age interval indicated.

on an elementary life-table formula.[4] The risks computed for close and distant relatives in the proband and in the control series are found to be of the same magnitude, thus indicating no significant difference between the proband and the control series with regard to the risk of dying from cancer.

Table 4 shows the number of relatives in the control and proband series with different forms of cancer. None of the main groups differ significantly. The age at death from cancer may be read from table 3, which shows that in this respect, too, the proband and the control series are practically identical.

TABLE 4.—*Type and site of malignant growths in relatives of probands and controls*

| Type and site of primary cancer | Relatives of probands | Relatives of controls |
|---|---|---|
| Intracranial tumors | 5 | 5 |
| Mesodermal tumors: | | |
| Leukemia | 9 | 4 |
| Hodgkin's disease | 1 | 1 |
| Sarcomas | 9 | 8 |
| Carcinomas: | | |
| Upper alimentary tract | 3 | 3 |
| Esophagus | 3 | 11 |
| Stomach | 29 | 22 |
| Intestine | 8 | 7 |
| Rectum, anus | 7 | 10 |
| Pancreas, bile duct, and gall bladder | 5 | 2 |
| Upper respiratory tract | 1 | 1 |
| Lungs | 4 | 1 |
| Thyroid gland | 0 | 5 |
| Breast | 7 | 10 |
| Urinary tract | 5 | 6 |
| Uterus | 10 | 7 |
| Ovary | 3 | 3 |
| Vulva | 0 | 1 |
| Skin | 1 | 0 |
| Questionable | 25 | 23 |
| Total | 135 | 130 |

Hence, the present study did not reveal any significant difference between the proband and control series with respect to the occurrence of intracranial tumors, of cancer in general, of cancer of special sites, nor with regard to the age at which the cancer manifests itself (expressed by the age at death from cancer).

[4] In the original formula: $R_{80}=100-100\,e^{-\int_0^{80}\mu_x dx}$ where $e$ denotes the base of the natural logarithm and $\mu$ is the death intensity at a given age, the integration over the period from age 0 to age 80 is approximated as follows: $\int_0^{80} \approx \Sigma(A/L)$, where A is the number of deaths from cancer in a given age interval, and L is the number of persons having passed this interval. The variance is estimated from the expression: Variance of computed $R_{80} \approx (100-R_{80})^2\Sigma(A/L^2)$.

### *Twins*

Three of the probands were twins. In all cases the co-twins were healthy.

| | | | |
|---|---|---|---|
| (1) | ■ died at 40 years | □ 46 years | monozygotic, discordant |
| (2) | ■ died at 53 years | □ died at 55 years | dizygotic, discordant; co-twin died from chronic cor pulmonale |
| (3) | ■ died at 53 years | ○ 55 years | dizygotic, discordant |

## Discussion

The present findings do not support the statement that hereditary factors play a major role in the etiology of glioblastoma. This applies to factors influencing the development of malignant growths on the whole, as well as to factors governing the localization of tumors, since neither the total cancer risk nor the risk of intracranial tumor is higher among the relatives of the probands than among those of the controls.

On the basis of the reported cases of familial glioblastomas and of the concordant occurrence of glioblastomas in identical twins, Koch (*7*) concluded that in certain cases glioblastoma is hereditary. He even goes so far as to advance the hypothesis that the hereditary cases are due to an inherited "gliadysplasie," also said to be demonstrable microscopically in tissue beyond the tumor proper. A perusal of the literature, however, shows that the "familial" occurrence of glioblastoma has been reported in only 5 cases (*6*, *9–11*). In all the other cases the nature of the tumor is questionable in 1 or both cases or else only 1 of the relatives had a glioblastoma, whereas the other had an intracranial tumor of a different nature. In a disease which is so common, relatively, as glioblastoma, familial cases must be expected now and then but it does not necessarily follow that heredity plays a role.

Glioblastoma in twins which were probably monozygotic has been reported only 5 times (*12–16*). Real concordance, however, was present in only 1 of the cases (*13*), in which both partners had gliomas. The case histories and microscopic reports ("glioma"), however, do not give any clear evidence that the tumors were glioblastomas. Thus, the literature on twins does not support the assumption that glioblastomas may be hereditary.

The distribution of cancer among the relatives in our proband series was, with respect to nature and localization, rather close to the distribution of cases reported to the Cancer Registry (*cf.* Brøbeck (*1*), pp. 64, 65). The only exception is afforded by mesodermal tumors. Their relative frequency among secondary-cancer cases in our series was significantly higher than the relative frequency listed in the Cancer Registry (19 out of 135 cases of cancer in our proband series as compared with 1,013 out

of 25,735 cases reported to the Cancer Registry). However, in the absence of any significant difference between our proband and control series for the occurrence of mesodermal tumors, this probably means merely that mesodermal tumors—no doubt primarily leukemias—are often not reported to the Cancer Registry.

## Conclusion

The present series indicates that inherited factors do not play any major role in the etiology of glioblastoma.

## Summary

Among 1,744 relatives of 169 patients with histologically verified glioblastomas, the authors found 5 cases of intracranial tumors and 129 cases of cancer at other sites. For purposes of comparison, the authors studied 1,625 relatives of 170 controls of the same sex and age distribution as the proband series, and found 5 cases of intracranial tumor and 125 of cancer of other sites. On the basis of these findings it is concluded that inherited factors play no major etiologic role in glioblastomas and that the latter bear no genetic relation to other types of cancer.

## References

(*1*) Brøbeck, O.: Heredity in Cancer Uteri. A Genetical and Clinical Study of 200 Patients with Cancer of the Cervix Uteri. Universitetsforlaget, Århus, 1949.

(*2*) Jacobsen, O.: Heredity in Breast Cancer. Copenhagen, Arnold Busck, Århus, 1946.

(*3*) Videbæk, Å.: Heredity in Human Leukemia and Its Relation to Cancer. Copenhagen, Arnold Busck, 1947.

(*4*) Videbæk, Å., and Mosbech, J.: The etiology of gastric carcinoma elucidated by a study of 302 pedigrees. Acta med. scandinav. 149: 137–159, 1954.

(*5*) Kemp, T.: Heredity in human cancer. Brit. J. Cancer 2: 144–149, 1948.

(*6*) Koch, G.: Erbliche Hirngeschwülste. Ztschr. menschl. Vererb.- u. Konstitutionslehre 29: 400–423, 1949.

(*7*) ———: Beitrag zur Erblichkeit der Hirngeschwülste. Acta genet. med. et gemel. 3: 170–191, 1954.

(*8*) Nyholm, M., and Helweg-Larsen, H. F.: On the computation of morbid risk (disease expectancy). Acta genet. et statistica med. 5: 25–38, 1954.

(*9*) Bender, W., and Panse, F.: Familiäres Gliom (Zur Genetik der Gliome). Monatschr. Psychiat. u. Neurol. 38: 253–258, 1932.

(*10*) Hoffmann, H.: Gehirntumoren bei zwei Geschwistern. Ein Beitrag zur Vererbung der Geschwülste. Ztschr. f. d. ges. Neurol. u. Psychiat. 51: 113–123, 1919.

(*11*) Zülch, K. J.: 1951. *Cited by* Koch, Acta genet. med. et gemel. 3:170–191, 1954.

(*12*) Geyer, H., and Pedersen, O.: Diskordantes Auftreten von Hirntumoren bei erbgleichen Zwillingen. Zentralbl. f. Neurochir. 3: 53–62, 1938.

(*13*) Joughin, J. L.: Coincident tumor of the brain in twins. Arch. Neurol. & Psychiat. 19: 945–948, 1938.

(*14*) Hoppe, H. J.: Diskordantes Auftreten von Hirntumoren bei erbgleichen Zwillingen. Zentralbl. f. Neurochir. 12: 34–36, 1952.

(*15*) Kuhnen, B.: Beobachtungen an sieben Zwillingspaaren mit Hirntumoren. Acta genet. med. et gemel. 2: 407–430, 1953.

(*16*) Versluys: 1934. *Cited by* Koch, Acta genet. med. et gemel. 3:170–191, 1954.

# Quantitative Studies of the Local Cellular Response to Isoantigens [1,2]

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Studies of the changes in the cell population of the peritoneal fluid of mice following an intraperitoneal injection of nontoxic antigenic material have recently been reported (*1–4*). In mice not previously sensitized to the antigenic material, there was an immediate and transient neutrophilia, a slight eosinophilia, and a gradual increase in the number of mononuclear cells, depending upon the type of antigen injected. However, in mice which had been previously sensitized to the injected antigen, differences in response were noted both in the mononuclear and eosinophil cells. There was a significant increase in the number of these cells, as well as a different pattern of response. A local eosinophilia between the 4th and 10th days was found to occur whenever an antigen was reinjected into sensitized animals. These cells therefore appeared to be involved in the immune responses of the animals.

This paper reports additional information concerning the specificity of the eosinophil response in the immune reactions of the mouse. A comparison was made of the local cellular responses following single and multiple injections of native tissue extracts (recipient and donor mice were of the same inbred stock) with the responses obtained following similar injections of an isoantigen (tissue extracts from donor mice which were genetically different from the recipient.) Studies were also made to determine whether animals which had successfully overcome a tumor homograft would show signs of sensitization when challenged with a nontumorous tissue extract derived from the strain of origin of the tumor.

## Materials and Procedure

The materials injected were extracts of frozen-dried spleen from A/Sn, $BBF_1$, and B10.D2 (*see* below) mice and transplantable tumor cells from

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[3] Public Health Service Special Research Fellow of the National Cancer Institute, now at Brookhaven National Laboratory, Upton, N. Y.
[4] The authors would like to take this occasion to thank Dr. U. Wenck and Dr. A. Kandutsch for their help and suggestions.

strain A sarcoma I (SaI). The spleen was harvested and frozen-dried, and prior to use, was homogenized with physiologic saline in a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 900 × g, the supernatant was decanted, and the sediment was then re-extracted twice with saline. The supernatants were pooled and adjusted to a concentration of 27 mg. dry weight per ml. The SaI tumor cells were harvested and a suspension composed primarily of single cells was made (*5*).

The recipient mice consisted of A/Sn, the hybrid $BBF_1$, and C57BL/10 × DBA/2 (hereafter referred to as B10.D2) (*2,6*). Only males from 3½ to 6 months of age were used. They were housed 1 to 3 animals per cage and fed *ad libitum* on Purina laboratory chow. Two days before the peritoneal-fluid samples were taken, the cages of mice were placed in a darkened cabinet maintained at 37° C., and care was taken not to disturb the animals before sampling.

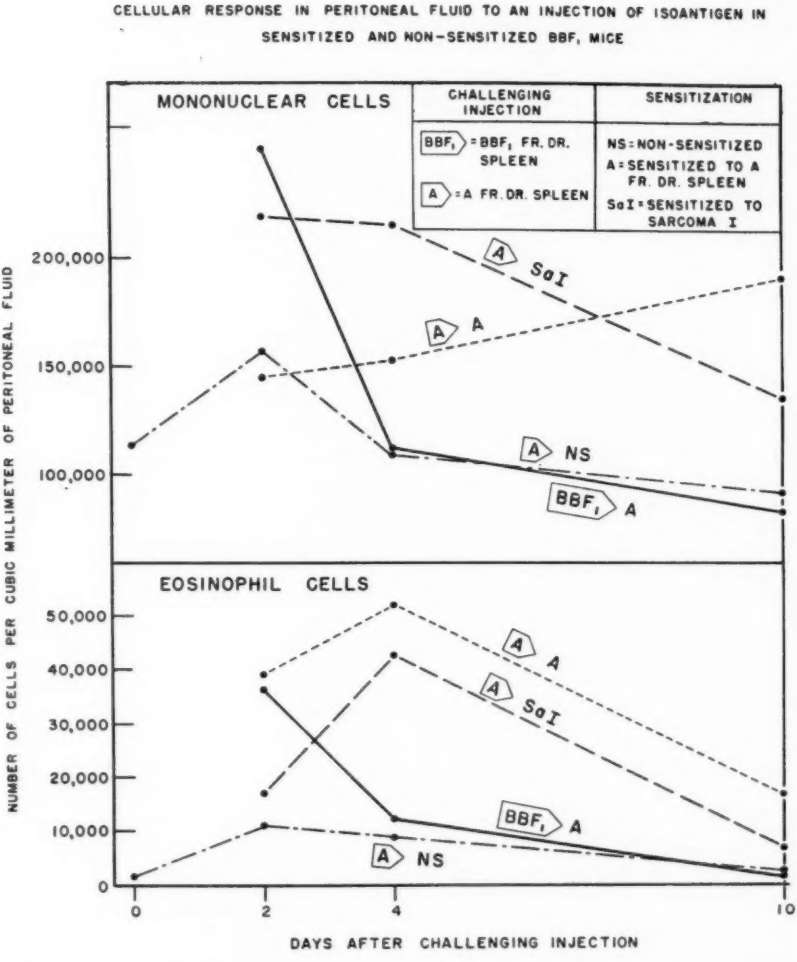
In a group of 115 mice, the cellular content of the peritoneal fluid was determined following intraperitoneal injections of 0.2 ml. of splenic-tissue extract. The animals receiving multiple injections of the extract were given 5 subcutaneous injections of 0.2 ml. each on days 1, 2, 3, 8, and 15 and a final intraperitoneal injection on the 22nd day.

A second group consisting of 13 mice was inoculated subcutaneously with living SaI cells, approximately 1 million cells per mouse in 0.5 ml. of Ringer's solution. Eleven days later there was small to medium tumor growth in all animals, and by day 25 these tumors had completely regressed. On day 25 and day 40 two smaller booster injections of SaI cells were given but no tumor growth was observed. One week after the last booster injection, these mice were challenged with an intraperitoneal injection of 0.2 ml. of an extract prepared from spleens of nontumorous animals of the strain of origin of the tumor (strain A).

The peritoneal fluid was withdrawn through a small ventral midline incision in a standard red-cell pipette and diluted 1:200 for chamber counts of total and eosinophil cells. The diluting fluid for total cells was a 30% acetone solution plus toluidine blue. The eosinophil diluent consisted of a 15% acetone solution plus phloxin B. Care was taken to count cells in the center of the counting chamber within 5 to 10 minutes after loading to avoid concentration of the cells by evaporation of the acetone. Since this study was done, propylene glycol has been found to be a more satisfactory diluent for total-cell counts of peritoneal fluid (*2*). Two peritoneal-fluid smears were made for each animal, and differential counts were determined by counting at least 100 cells on each slide.

## Results and Discussion

The data obtained in this experiment are summarized in table 1 and text-figure 1. It may be seen that on the second day after all intraperitoneal injections a neutrophilia occurred as well as an increase in mononuclear and eosinophil cells. The neutrophils are rarely found in the fluid of the noninflamed peritoneal cavity, but immediately following an injection they appear in large numbers (*2,3*). They usually begin to




TEXT-FIGURE 1.—Response of cells in peritoneal fluid to an injection of isoantigen in sensitized and nonsensitized $BBF_1$ mice.

diminish in number after 24 hours and are found only occasionally after the 4th day. However, to get a more detailed picture of the response of this cell, additional periods between 1 and 24 hours would have to be studied, which was not done in this experiment.

The mononuclear cells increased in number on the second day following all injections. The amount of increase was similar regardless of whether the material injected was an isoantigen or a native tissue extract, or whether the animal had been previously injected with that material. By the 4th and 10th days the number of these cells decreased to approximately normal levels in all animals, with the exception of those which were

TABLE 1.—*Cellular responses in peritoneal fluid following an intraperitoneal injection of tissue extracts in sensitized and nonsensitized mice and in mice previously inoculated with tumor cells*

| Recipient animals | | Tissue extract* | | Cellular response (mean ± S.E./mm.³) | | | |
|---|---|---|---|---|---|---|---|
| Strain | No. of mice | Pretreatment (5 subcutaneous injections) | Final intraperitoneal injection | Days after final injection | Eosinophils | Mononuclear cells | Neutrophils |
| $BBF_1$ | 11 | None | None | 0 | 1,840 ± 477 | 112,100 ± 10,789 | 0 |
| | 4 | None | $BBF_1$ | 2 | 6,300 ± 750 | 182,421 ± 41,112 | 3,066 ± 1,079 |
| | 5 | | | 4 | 3,370 ± 1,387 | 77,423 ± 15,300 | 2,583 ± 898 |
| | 5 | | | 10 | 1,160 ± 431 | 69,652 ± 3,254 | 1,866 ± 1,643 |
| | 5 | | A | 2 | 11,130 ± 1,593 | 157,620 ± 12,805 | 4,462 ± 1,457 |
| | 5 | | | 4 | 8,810 ± 1,831 | 109,858 ± 16,350 | 331 ± 216 |
| | 5 | | | 10 | 2,190 ± 604 | 92,151 ± 7,625 | 119 ± 120 |
| | 5 | $BBF_1$ | $BBF_1$ | 2 | 11,570 ± 1,788 | 238,527 ± 14,613 | 4,142 ± 1,709 |
| | 5 | | | 4 | 7,490 ± 3,146 | 84,091 ± 6,730 | 284 ± 120 |
| | 5 | $BBF_1$ | A | 2 | 30,285 ± 11,710 | 217,568 ± 24,871 | 6,451 ± 2,936 |
| | 4 | | | 4 | 13,342 ± 3,621 | 125,325 ± 24,497 | 697 ± 697 |
| | 4 | | | 10 | 2,380 ± 1,706 | 87,405 ± 7,579 | 455 ± 305 |
| | 5 | A | $BBF_1$ | 2 | 36,264 ± 19,394 | 251,147 ± 15,485 | 6,465 ± 2,589 |
| | 5 | | | 4 | 12,240 ± 3,524 | 113,286 ± 8,666 | 622 ± 337 |
| | 5 | | | 10 | 1,380 ± 404 | 83,764 ± 8,454 | 0 |
| | 5 | A | A | 2 | 39,123 ± 10,312 | 146,014 ± 40,182 | 8,915 ± 1,198 |
| | 5 | | | 4 | 51,775 ± 13,884 | 150,332 ± 11,673 | 165 ± 165 |
| | 4 | | | 10 | 17,250 ± 3,033 | 191,250 ± 35,317 | 431 ± 273 |
| | 4 | SaI tumor cells | A | 2 | 17,312 ± 4,300 | 219,353 ± 36,450 | 26,603 ± 14,882 |
| | 4 | | | 4 | 42,538 ± 16,775 | 216,265 ± 34,513 | 308 ± 307 |
| | 5 | | | 10 | 7,040 ± 1,098 | 135,746 ± 5,004 | 142 ± 142 |
| B10.D2 | 4 | None | B10.D2 | 4 | 8,212 ± 2,716 | 123,042 ± 4,646 | 418 ± 247 |
| | 5 | | A | 4 | 9,740 ± 2,918 | 128,223 ± 11,442 | 314 ± 221 |
| | 5 | A | B10.D2 | 4 | 4,550 ± 1,186 | 134,108 ± 21,189 | 1,123 ± 510 |
| | 5 | | A | 4 | 20,975 ± 8,256 | 143,823 ± 16,052 | 504 ± 215 |
| A | 5 | A | A | 4 | 3,890 ± 872 | 60,232 ± 5,694 | 113 ± 70 |

*All injections were frozen-dried spleen extracts of the strain indicated except for the 13 $BBF_1$ mice which received 3 injections of living SaI tumor cells during the period of sensitization.

reinjected with the isoantigen to which they had been previously sensitized. For example, the $BBF_1$ mice which had been sensitized and challenged with strain A splenic-tissue extract showed a progressive increase in the number of mononuclear cells over the period of the experiment. The animals which had been sensitized with sarcoma I cells and challenged with A splenic-tissue extract had a mononucleosis on the 4th day which decreased by the 10th day. Thus, the animals which had been sensitized and reinjected with an isoantigen had a prolonged period of mononucleosis compared with animals injected with native tissue extract. It is possible that certain types of mononuclear cells were responding specifically to the reinjected antigen. However, all gradations in morphology from one cell type to another were observed, and we were unable to differentiate clearly among the lymphocytes, plasma cells, histiocytes, monocytes, etc., with the staining techniques used. The problems of differentiating these cells have been discussed in detail by Bloom (*7,8*).

The eosinophil cells markedly increased in number in the peritoneal fluid following an injection of antigen, but there appeared to be 2 different types of reactions. When the intraperitoneal injection consisted of native tissue extract or when the isoantigen was injected into mice not previously sensitized to that antigen, an eosinophilia occurred on the second day after injection. This eosinophilia was relatively mild, subsided by the 4th day, and was completely absent by the 10th day. However, when the intraperitoneal injection consisted of the isoantigen to which the animal had been previously sensitized, a greater and more prolonged eosinophilia occurred which reached a peak at 4 days and was still evident on the 10th day. Likewise, the animals which had previously been injected with strain A tumor cells (SaI) showed a similarly prolonged accumulation of eosinophils following an injection of strain A tissue extract. However, the 4- and 10-day eosinophilia did not occur following reinjection of native tissue extract. For example, repeated injections of strain A splenic-tissue extract resulted in a marked accumulation of eosinophils 4 days after the final injection in $BBF_1$ and B10.D2 mice but not in strain A/Sn animals. This indicates that the eosinophilia is related to immune mechanisms of the mice and not to nonspecific responses produced by injections of tissue. These data confirm and extend previous observations in which foreign proteins, toxoids, complex polysaccharides (pollen), and parasitic extracts produced characteristic local eosinophil-response curves when injected into animals previously immunized against the antigen (*1–4*).

The role of various cells which accumulate around tissue grafts, tumors, and pathogens has been largely neglected in recent years. This has also been noted by Felix and Dalton (*9*), who recently emphasized the need for study of normal peritoneal-fluid cells in studies of ascites-tumor growth. The experiments in this paper demonstrate that the cell changes which occur in the peritoneal fluid following an intraperitoneal injection vary, depending upon the state of host immunity. The specificity and extent of the eosinophilia induced by isoantigens reported in this paper tend to emphasize the importance of these cells in host resistance. Interesting

observations along this line have been made by Casey (*10*). This is emphasized even further in recent work describing a local accumulation of eosinophil cells around tissue homografts about to slough off (*11,12*). It is possible that further studies may enable the pathologist to determine the state and degree of host resistance by determining the nature of the local cellular response. This could be done quantitatively, not only in the peritoneal fluid but also in other body fluids.

## Summary and Conclusions

1) This paper describes the local cellular responses to injections of isoantigens in sensitized and nonsensitized mice and in mice which had previously overcome tumor transplants.

2) It was observed that injections of isoantigens into nonsensitized mice produced a transient neutrophilia, eosinophilia, and mononucleosis of the peritoneal fluid. This response subsided by the 4th day and disappeared by the 10th day after injection.

3) In animals which were challenged with the isoantigen to which they had been previously sensitized there was also a transient neutrophilia. However, there was a marked accumulation of eosinophils, reaching a peak on the 4th day and still present 10 days after injection. An increase in the number of mononuclear cells was also observed on the 4th and 10th days.

4) Repeated injections of living sarcoma I cells into $BBF_1$ mice sensitized the animals to the isoantigen of the strain of origin of the tumor, as determined by the local eosinophil response.

5) The accumulation of eosinophils between 4 and 10 days did not occur following repeated injections of tissue extracts prepared from mice of the same genetic constitution as the recipient. This indicates that the response is specific for antigenic materials and must be related to immune reactions of the animal.

6) It was suggested that further quantitative studies of the local cell population around grafts, tumors, and pathogens would give indications of the state and degree of host resistance.

## References

(*1*) Speirs, R. S.: Physiological approaches to an understanding of the function of eosinophils and basophils. Ann. New York Acad. Sc. 59: 706–731, 1955.

(*2*) Speirs, R. S., and Dreisbach, M. E.: Quantitative studies of the cellular responses to antigen injections in normal mice. Blood 11: 44–55, 1956.

(*3*) Speirs, R. S., Wenck, U., and Dreisbach, M. E.: Quantitative studies of the cellular responses to antigen injections in adrenalectomized mice. Blood 11: 56–70, 1956.

(*4*) Speirs, R. S., and Wenck, U.: Eosinophil response to toxoids in actively and passively immunized mice. Proc. Soc. Exper. Biol. & Med. 90: 571–574, 1955.

(*5*) Snell, G. D.: A cytosieve permitting sterile preparations of suspensions of tumor cells for transplantation. J. Nat. Cancer Inst. 13: 1511–1515, 1953.

(*6*) ———: Isogenic resistant (IR) lines of mice. Transplant. Bull. 2: 6–8, 1955.

(*7*) Bloom, W.: Lymphatic tissue; lymphatic organs. *In* Handbook of Hematology (Downey, H., ed.). New York, Paul B. Hoeber, Inc., 1938, Section 19.

(8) BLOOM, W.: Discussion of paper by James E. Kindred "Quantitative studies on lymphoid tissues." Ann. New York Acad. Sc. 59: 754–756, 1955.

(9) FELIX, M. D., and DALTON, A. J.: A phase-contrast microscope study of free cells native to the peritoneal fluid of DBA/2 mice. J. Nat. Cancer Inst. 16: 415–445, 1955.

(10) CASEY, A. E., and DRYSDALE, G. R.: Hereditary eosinophile levels in the acquired resistance of the rabbit to the Brown-Pearce tumor. (Abstract.) Cancer Res. 7: 728, 1947.

(11) ROGERS, B. O., CONVERSE, J. M., TAYLOR, A. C., and CAMPBELL, R. M.: The eosinophile in human skin homografting. Proc. Soc. Exper. Biol. & Med. 82: 523–526, 1953.

(12) ALLEN, H. L., WILLIAMS, R. D., LOVINGOOD, C. G., and ELLISON, E. H.: The effect of donor skin desensitization and ACTH on survival of skin homografts in rabbits. Ann. Surg. 135: 239–244, 1952.

# A Rapid Quantitative Hematocrit Method for Measuring Increase in Cell Population of Strain L (Earle) Cells Cultivated in Serum-Free Nutrient Solutions [1,2]

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The nutritional needs of the single-cell line of strain L mouse fibroblasts (*1,2*) have been the subject of numerous investigations (*3–17*). Estimation of the proliferation of cultures of this strain of cells has been made by means of (*a*) nuclear counts (*4,18–22*), (*b*) cell counts (*3*), (*c*) nucleic acid estimations (*23*), or (*d*) by rating on an arbitrary scale (*15,17*). The nuclear-count method has the disadvantage of requiring special and somewhat elaborate apparatus. For both nuclear and cell counts, cultures must be handled individually, and when several cultures are to be subjected to counts, the aggregate effort is tedious and time-consuming. The method to be described here requires only simple apparatus and has the advantage that several estimations can be run concurrently. It provides a quantitative measure of the size of the cell population with a precision consistent with the amount of variation to be expected in the culture system. The method is similar in principle to one briefly described by Gemmill, Gey, and Austrian (*24*) for measuring the volume of cells from roller-tube cultures of the Walker rat sarcoma 319. The technique of these investigators was similar to that described here in that they employed trypsin digestion and centrifuged the cells in a hematocrit tube.

## Materials and Methods

Stock cultures of strain L cells (A929-144-20) were obtained in December, 1953, through the courtesy of Dr. W. R. Earle. They had been grown at the National Cancer Institute in a medium of chick-embryo extract, horse serum, and balanced salt solution in a proportion of 20:40:40. For the first 4 months in our laboratory, stock cultures were maintained in Carrel D-3.5 flasks in a similar medium (*25*). Thereafter, by substituting a defined nutrient (*15,16*) for the balanced salt solution, it became possible to reduce the concentration of chick-embryo extract to 10 or

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[3] With the technical assistance of Mrs. M. A. Dunbar.

5 percent and the concentration of horse serum to 20 or 10 percent. From October, 1954, embryo extract was entirely omitted and all stock cultures were maintained in media containing 90 or 95 percent of a synthetic nutrient (ML 6) supplemented with 10 or 5 percent of rabbit serum instead of horse serum. The medium containing 10 percent rabbit serum was designated ML 94/4, that containing 5 percent rabbit serum, ML 97 (*16*). Each Carrel flask contained 1 ml. of nutrient medium.

The experimental cultures for which this quantitative growth assay was designed were grown in media containing no serum or embryo extract; instead they contained a commercial peptone (Difco Bacto-Peptone) and a protein (Armour's bovine serum albumin, fraction V). Medium ML 192/2 is the prototype of these media and is described in the accompanying paper (*16*). Cultures for assay were always prepared from cells which had been maintained in ML 192/2 for several passages, usually for months. Cells from the parent cultures were scraped off the floor of the flask with a pipette with a curved tip, and the cells were dispersed by rapidly and repeatedly taking up and ejecting the suspension with the pipette. The cultures were made from the well-stirred cell suspensions, using an equal number of drops from a single pipette for each flask in the series. The inocula varied from one series to another, but fell within the range of 160,000 to 400,000 cells per flask.

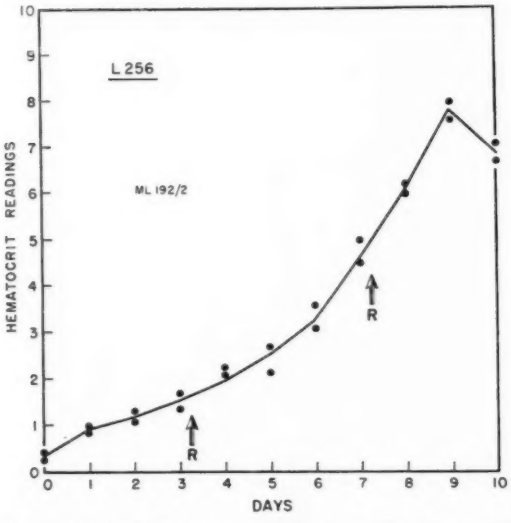
The cell population was measured by determining the volume of cells contained in 1 Carrel flask with a Van Allen hematocrit (*26*). This microhematocrit has a capillary stem and a bulb and its total capacity is slightly more than 1 ml. Thus, it conveniently contains the entire contents of 1 flask. The stem is graduated in 100 parts, and the length of the graduated section is $60.15 \pm 0.12$ mm. (standard error of the mean). The internal bore approximates 0.50 mm. in diameter. A check calibration on 24 hematocrits (supplied by A. H. Thomas Co., Philadelphia) demonstrated that the bore is of a satisfactory uniformity ($0.50 \pm 0.02$ mm.) to give reproducible readings.

In preparation for the assay, the cells were detached from the glass by introducing into each flask, without removing the nutrient, 0.2 ml. of a 0.25 percent solution of trypsin (1:300; Nutritional Biochemicals, Inc.) in 0.8 percent NaCl, 0.04 percent KCl, and 0.02 percent $Na_2HPO_4$. The flask was allowed to stand for about 1 minute and then shaken. This treatment is sufficient to detach all the cells. (A longer digestion time is necessary in media containing serum.) The cell suspension was transferred as completely as possible to a small siliconed shell vial and from there it was taken up quantitatively in the hematocrit, which was also siliconed. The hematocrit was closed with a spring clip (*27*) and centrifuged at 2800 r.p.m. for 30 minutes in an International centrifuge, size 1, Model SBV, with an 8-place head (#225) and metal shields (#303), using a ½-inch Gooch tubing rubber liner for each shield. The length of the column of packed cells was read on the graduated stem.

### Evaluation of Results

To assess the reproducibility of the results, readings were made on 2 series each of 16 replicate cultures. In the first series, 8 readings at 4 days gave $4.33 \pm 0.21$ and 8 readings at 7 days gave $6.32 \pm 1.25$. In the second series, with a smaller initial inoculum, 8 readings at 4 days gave $3.53 \pm 0.31$ and 8 readings at 7 days gave $5.61 \pm 0.62$.

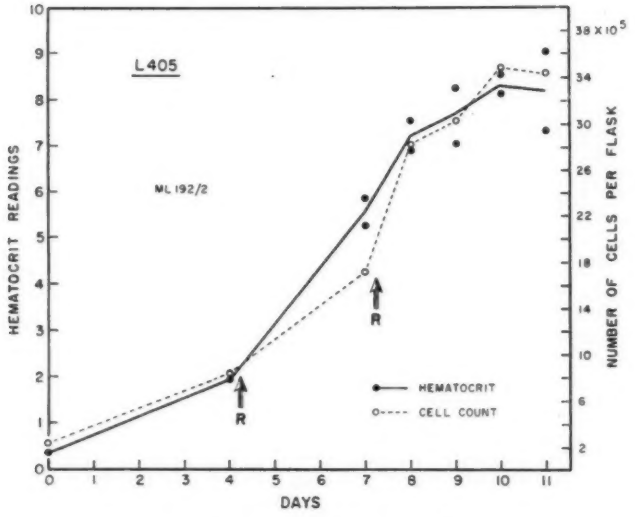
The quantity measured here (packed-cell volume) does not, of course, measure "growth" in terms of the same variables as measurement of cell or nuclear number. Text-figure 1 shows a typical response of a series of replicate cultures in ML 192/2 in terms of cell volume over a period of 10 days with nutrient renewal at 3 and 7 days. A comparison of hematocrit readings with hemocytometer counts (*3*) was made in several series of cultures. Text-figure 2 shows such a comparison.



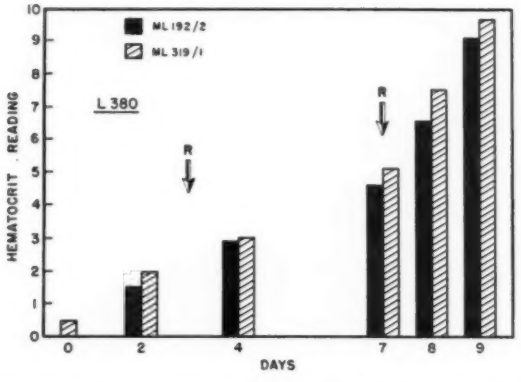

TEXT-FIGURE 1.—Packed-cell volume of replicate cultures in medium ML 192/2. Daily duplicate hematocrit readings. Nutrient medium renewed at 3 and 7 days (*R*).

In general, the agreement between the 2 methods is very satisfactory over the period 0 to 9 days, *i.e.*, for readings up to 7.5 or counts up to $3.0 \times 10^6$ cells. Variation between replicates increases as the cell population approaches the maximum that 1 ml. of nutrient will support. There appears to be a tendency, as the cells become crowded after active proliferation, for the average cell size to decrease (figs. 1 and 2 and text-fig. 1). For the study of growth rates in rapidly growing cultures, however, this variability is unimportant, because an experimental period of 7 days, during which at least tenfold increases in cell population may take place, is usually sufficient.

In the particular culture system for which this method was worked out, namely, strain L cells in medium ML 192/2, growth is rapid, and




TEXT-FIGURE 2.—Comparison between hematocrit readings and cell counts in replicate cultures in medium ML 192/2. Nutrient medium renewed at 4 and 7 days (*R*).



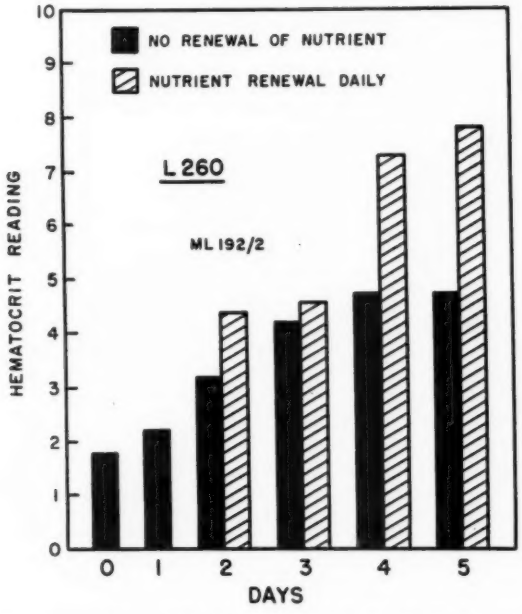

TEXT-FIGURE 3.—Comparison of growth of strain L cells in 2 serum-free nutrient media, ML 192/2 and ML 319/1. Media renewed at 3 and 7 days (*R*).

measurements cannot be made beyond 10 days. The upper limit of cell population which can be measured happens, fortuitously, to be, for these cells and probably for others, close to the maximum that a Carrel D-3.5 flask will support in 1 ml. of nutrient. The maximum population might be reached at a different time with other cells or with another nutrient.

An example of the use of the method is shown in text-figure 3. This represents a comparison of medium ML 192/2 with ML 319/1, the latter differing from ML 192/2 in the substitution of certain anions (Cl′ for

$SO_4''$ and $NO_3'$). There is a small but probably significant difference between these 2 media.

The growth response of replicate cultures over a period of 5 days with (*a*) no renewal of the nutrient and (*b*) daily replenishment with fresh ML 192/2 is shown in text-figure 4. Daily renewal of nutrient clearly results in continuation of growth after the cultures without renewal of medium have reached a stationary state.




TEXT-FIGURE 4.—Comparison of the growth of strain L cells in ML 192/2 with (*a*) no renewal of nutrient and (*b*) daily renewal.

## Conclusion

The growth of replicate tissue cultures of strain L cells in serum-free nutrient solutions can be evaluated simply and rapidly by serial measurements of the total volume of cells from 1 Carrel flask, using a Van Allen microhematocrit.

## References

(*1*) EARLE, W. R.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(*2*) SANFORD, K. K., EARLE, W. R., and LIKELY, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1948.

(3) CAILLEAU, R., and KIRK, P. L.: The influence of various culture media on the growth of Earle's strain L cells and chick-heart fibroblasts *in vitro*. J. Nat. Cancer Inst. 15: 295–303, 1954.

(4) EAGLE, H.: The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. Biol. Chem. 214: 839–852, 1955.

(5) ———: The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. J. Exper. Med. 102: 595–600, 1955.

(6) ———: Utilization of dipeptides by mammalian cells in tissue culture. Proc. Soc. Exper. Biol. & Med. 89: 96–99, 1955.

(7) ———: Nutrition needs of mammalian cells in tissue culture. Science 122: 501–504, 1955.

(8) EARLE, W. R., SANFORD, K. K., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The influence of inoculum size on proliferation in tissue cultures. J. Nat. Cancer Inst. 12: 133–153, 1951.

(9) EARLE, W. R., SCHILLING, E. L., BRYANT, J. C., and EVANS, V. J.: The growth of pure strain L cells in fluid-suspension cultures. J. Nat. Cancer Inst. 14: 1159–1171, 1954.

(10) EVANS, V. J., SHANNON, J. E., JR., BRYANT, J. C., WALTZ, H. K., EARLE, W. R., and SANFORD, K. K.: A quantitative study of the effect of certain chemically defined media on the proliferation *in vitro* of strain L cells from the mouse. J. Nat. Cancer Inst. 13: 773–783, 1953.

(11) EVANS, V. J., BRYANT, J. C., SANFORD, K. K., and EARLE, W. R.: Studies of certain defined media for testing proliferation in replicate tissue cultures. Ann. New York Acad. Sc. 58: 1012–1022, 1954.

(12) HEALY, G. M., FISHER, D. C., and PARKER, R. C.: Nutrition of animal cells in tissue culture. IX. Synthetic medium No. 703. Canad. J. Biochem. & Physiol. 32: 327–337, 1954.

(13) ———: Nutrition of animal cells in tissue culture. X. Synthetic medium No. 858. Proc. Soc. Exper. Biol. & Med. 89: 71–77, 1955.

(14) WAYMOUTH, C.: Some effects of inorganic phosphate and bicarbonate on cell survival and proliferation in chemically defined nutrient media. Biochem. J. 56: iv, 1954.

(15) ———: Simple nutrient solutions for animal cells. Texas Rep. Biol. & Med. 13: 522–536, 1955.

(16) ———: A serum-free nutrient solution sustaining rapid and continuous proliferation of strain L (Earle) mouse cells. J. Nat. Cancer Inst. 17: 315–327, 1956.

(17) WHITE, P. R.: An approach to chemically defined nutrients for strain L mouse cells. J. Nat. Cancer Inst. 16: 769–787, 1955.

(18) EVANS, V. J.: Preparation and handling of replicate tissue cultures for quantitative studies. Methods in Med. Res. 4: 221–225, 1951.

(19) EVANS, V. J., EARLE, W. R., SANFORD, K. K., SHANNON J. E., and WALTZ, H. K.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907–927, 1951.

(20) SANFORD, K. K.: Measurement of proliferation of tissue cultures by enumeration of cell nuclei. Methods in Med. Res. 4: 219–221, 1951.

(21) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E.: The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795. 1951.

(22) PARKER, R. C., HEALY, G. M., and FISHER, D. C.: Nutrition of animal cells in tissue culture. VII. Use of replicate cell cultures in the evaluation of synthetic media. Canad. J. Biochem. & Physiol. 32: 306–318, 1954.

(23) HEALY, G. M., FISHER, D. C., and PARKER, R. C.: Nutrition of animal cells in tissue culture. VIII. Desoxyribonucleic acid phosphorus as a measure of cell multiplication in replicate cultures. Canad. J. Biochem. & Physiol. 32: 319–326, 1954.

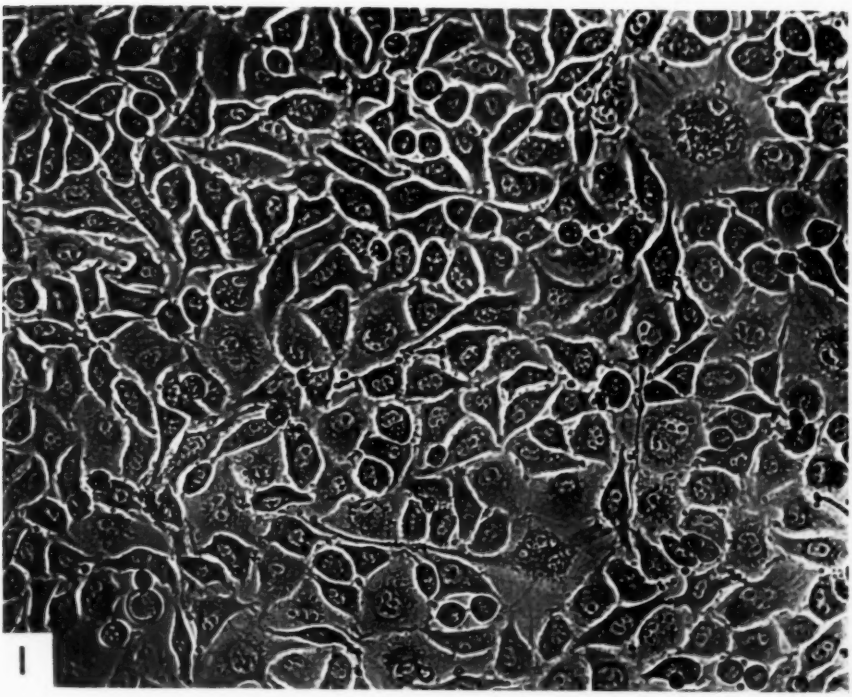
(*24*) GEMMILL, C. L., GEY, G. O., and AUSTRIAN, R.: The metabolism of tissue cultures of Walker rat sarcoma 319. Bull. Johns Hopkins Hosp. 66: 167–184, 1940.
(*25*) WAYMOUTH, C., and WHITE, P. R.: Filtration of embryo extract for tissue cultures. Science 119: 321–322, 1954.
(*26*) VAN ALLEN, C. M.: An hematocrit method. J.A.M.A. 84: 202–203, 1925.
(*27*) ———: Sealing device for hematocrit. J.A.M.A. 85: 2033, 1925.

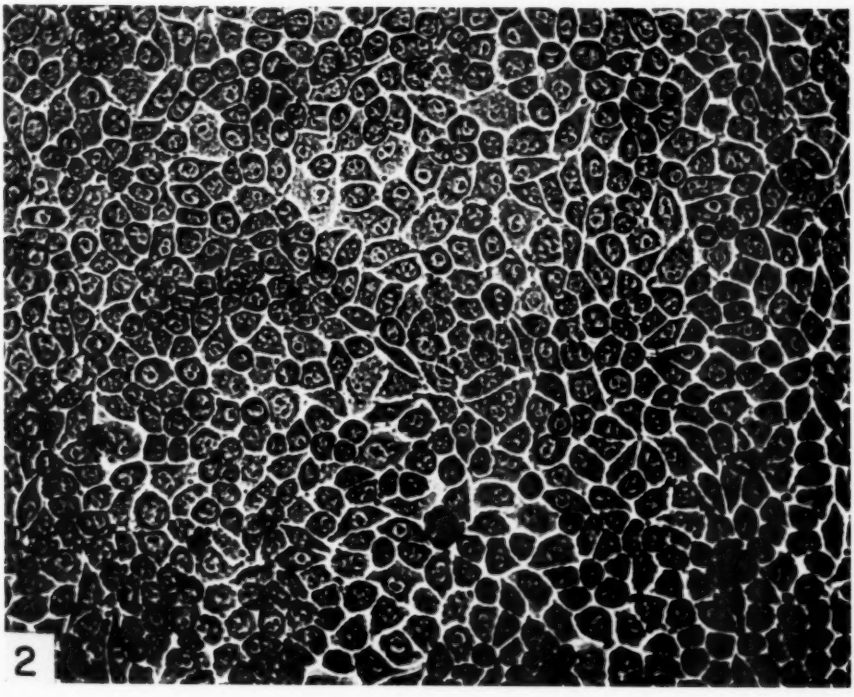
PLATE 19

**Cultures of strain L cells in ML 192/2 showing decrease in cell size. × 200**

FIGURE 1.—After 4 days.

FIGURE 2.—After 6 days.






393045—56——5

# A Serum-Free Nutrient Solution Sustaining Rapid and Continuous Proliferation of Strain L (Earle) Mouse Cells [1,2]

CHARITY WAYMOUTH,[3] *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

Strain L mouse cells (*1–3*) proliferate rapidly in the usual biological media, *e.g.*, chick-embryo extract, horse serum, and balanced salt solution in the proportion of 20:40:40. Similar rates of multiplication have been achieved in various derivative nutrients, containing (in place of whole embryo extract or horse serum) dialysates, ultrafiltrates, or high-molecular residues from these components (*4–11*). Fivefold to sixfold increases (*12*) or ninefold to tenfold increases (*13*) in cell number over a 7-day period have been reported for cultures of this strain in nutrients which are elaborate but fully synthetic.

Recently (*14*) some simple synthetic nutrient solutions were described, in one of which (solution C) strain L cells could be maintained in a state of survival without appreciable proliferative activity. When supplemented with serum, these solutions support rapid proliferation. Stock cultures have been maintained in this laboratory for more than a year in media similar to solution C, supplemented with 5 or 10 percent of rabbit serum, but containing no embryo extract. From these stocks, experimental cultures have been made to study the possibility of dispensing with the serum as well as the embryo extract.

The immediate objective of the present study was to define conditions within which proliferation could take place in a serum-free solution. The nutrient described below is one which fulfills this objective. The ultimate aim is to devise simple and chemically reproducible nutrient solutions capable of permitting cell proliferation.

## Materials and Methods

The method of maintaining stock cultures of strain L, clone 929, cells is described in the accompanying paper (*15*). The media in which the stock cultures were grown consisted of a synthetic nutrient ML 6, supplemented with 5 or 10 percent of rabbit serum. ML 6 differs from solution

[1] Received for publication April 27, 1956.

[2] Supported by a grant to Dr. P. R. White (CP-59) from the American Cancer Society, made upon recommendation of the Committee on Growth of the National Research Council.

[3] With the technical assistance of Mrs. M. A. Dunbar.

C (*14*) in containing half the concentration of dextrose, a quarter of the concentration of glycine, and a slightly different salt mixture. The composition of the resulting serum-containing media ML 97 and ML 94/4 is shown in table 1. A 6-day-old stock culture, from a line maintained 435 days in medium ML 97, is shown in figure 1.

TABLE 1.—*Serum-containing media ML 97 and ML 94/4*

| Stock solution | Materials | mg./100 ml. | Nutrient (*ml./100 ml.*) | Composition of ML 97 and ML 94/4 (*mg./100 ml.*) |
|---|---|---|---|---|
| $H_2O$ | $H_2O$ | | ML 97....57.0<br>ML 94/4..52.0 | |
| S/S1 | NaCl | 6,000 | | 600 |
| | KCl | 150 | 10.0 | 15 |
| | $Ca(NO_3)_2 \cdot 4H_2O$ | 200 | | 20 |
| | $MgSO_4 \cdot 7H_2O$ | 200 | | 20 |
| S/S2 | $Na_2HPO_4$ | 750 | | 30 |
| | $KH_2PO_4$ | 200 | 4.0 | 8 |
| S/S3 | $NaHCO_3$ | 5,600 | 4.0 | 224 |
| S/S4 | Dextrose | 10,000 | 2.5 | 250 |
| S/S5 | L-cystine | 15 | | 1.5 |
| | L-tyrosine | 40 | | 4.0 |
| | Glycine | 50 | | 5.0 |
| | L-lysine HCl | 160 | | 16.0 |
| | L-methionine | 50 | | 5.0 |
| | L-threonine | 75 | | 7.5 |
| | DL-valine | 125 | | 12.5 |
| | L-arginine HCl | 75 | 10.0 | 7.5 |
| | L-histidine HCl | 75 | | 7.5 |
| | L-proline | 50 | | 5.0 |
| | DL-isoleucine | 50 | | 5.0 |
| | β-phenyl-L-alanine | 50 | | 5.0 |
| | L-leucine | 50 | | 5.0 |
| | L-glutamic acid | 150 | | 15.0 |
| | L-aspartic acid | 60 | | 6.0 |
| | L-tryptophan | 40 | | 4.0 |
| | Phenol red, 0.5 ml. of 1.0% solution | | | |
| S/S6 | Thiamin HCl | 20 | | 0.5 |
| | Calcium pantothenate | 2 | | 0.05 |
| | Riboflavine | 2 | 2.5 | 0.05 |
| | Pyridoxine HCl | 2 | | 0.05 |
| | Folic acid | 0.4 | | 0.01 |
| | Biotin | 0.4 | | 0.01 |
| S/S7 | Hypoxanthine | 50 | 5.0 | 2.5 |
| | Glutamine | 100 | | 5.0 |
| Rabbit serum | | | ML 97.... 5.0<br>ML 94/4..10.0 | 5%<br>10% |

The experimental nutrient, designated ML 192/2, consisted of the components of ML 6, supplemented with the following:

1. Armour's bovine albumin, fraction V
2. Difco Bacto-Peptone

3. Ascorbic acid

   Cysteine

   Glutathione

These supplements replaced the serum of media ML 97 and ML 94/4. The composition of ML 192/2 is shown in table 2.

TABLE 2.—*Serum-free medium ML 192/2*

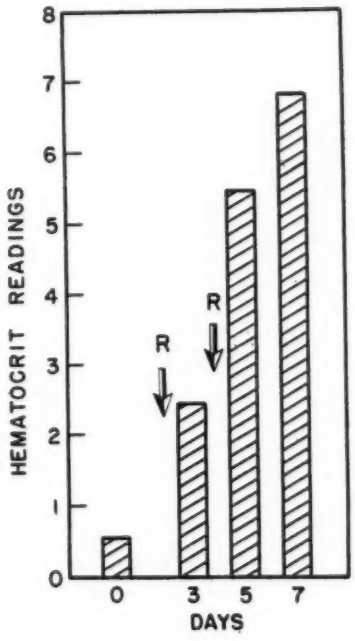
| Stock solution | Materials | mg./100 ml. | Nutrient (*ml./100 ml.*) | Composition of ML 192/2 (*mg./100 ml.*) |
|---|---|---|---|---|
| $H_2O$ | $H_2O$ | | 32. 0 | |
| S/S1 | NaCl | 6, 000 | | 600 |
| | KCl | 150 | 10. 0 | 15 |
| | $Ca(NO_3)_2 \cdot 4H_2O$ | 200 | | 20 |
| | $MgSO_4 \cdot 7H_2O$ | 200 | | 20 |
| S/S2 | $Na_2HPO_4$ | 750 | 4. 0 | 30 |
| | $KH_2PO_4$ | 200 | | 8 |
| S/S3 | $NaHCO_3$ | 5, 600 | 4. 0 | 224 |
| S/S4 | Dextrose | 10, 000 | 5. 0 | 500 |
| S/S5 | L-cystine | 15 | | 0. 75 |
| | L-tyrosine | 40 | | 2. 0 |
| | Glycine | 50 | | 2. 5 |
| | L-lysine HCl | 160 | | 8. 0 |
| | L-methionine | 50 | | 2. 5 |
| | L-threonine | 75 | | 3. 75 |
| | DL-valine | 125 | | 6. 25 |
| | L-arginine HCl | 75 | 5. 0 | 3. 75 |
| | L-histidine HCl | 75 | | 3. 75 |
| | L-proline | 50 | | 2. 5 |
| | DL-isoleucine | 50 | | 2. 5 |
| | β-phenyl-L-alanine | 50 | | 2. 5 |
| | L-leucine | 50 | | 2. 5 |
| | L-glutamic acid | 150 | | 7. 5 |
| | L-aspartic acid | 60 | | 3. 0 |
| | L-tryptophan | 40 | | 2. 0 |
| | Phenol red, 0.5 ml. of 1% solution | | | |
| S/S6 | Thiamin HCl | 20 | | 0. 5 |
| | Calcium pantothenate | 2 | | 0. 05 |
| | Riboflavine | 2 | 2. 5 | 0. 05 |
| | Pyridoxine HCl | 2 | | 0. 05 |
| | Folic acid | 0. 4 | | 0. 01 |
| | Biotin | 0. 4 | | 0. 01 |
| S/S7 | Hypoxanthine | 50 | 5. 0 | 2. 5 |
| | Glutamine | 100 | | 5. 0 |
| S/S8 | Ascorbic acid | 70 | | 1. 75 |
| | Cysteine | 300 | 2. 5 | 7. 78 |
| | Glutathione | 60 | | 1. 50 |
| S/S9 | Armour's bovine albumin, fraction V | 1, 000 | 5. 0 | 50. 0 |
| S/S10 | Difco Bacto-Peptone | 2, 000 | 25. 0 | 500. 0 |

The effectiveness of the nutrient ML 192/2 for increasing the cell population was assessed by the hematocrit method (*15*). These quantitative measurements were made at intervals beginning, usually, after cultures had been maintained for at least 4 weeks in ML 192/2.

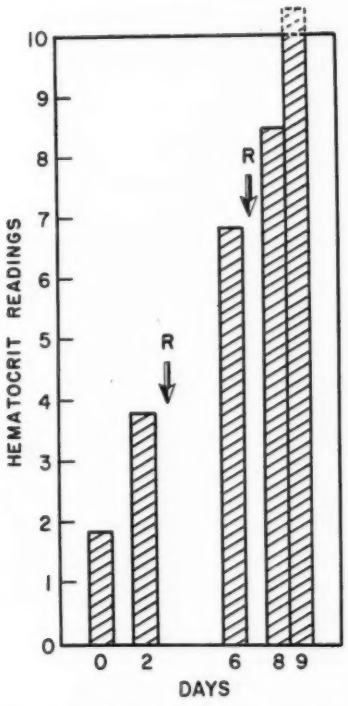
## Results

Cultures which had been in the test medium ML 192/2 for 28 days grew, over a period of 7 days, as shown in text-figure 1. With the relatively small inoculum used (hematocrit reading, H=0.55), there was a fivefold increase in cell population in 3 days, and by 7 days the increase was more than twelvefold. In another experiment (text-fig. 2), also using cells which had been 28 days in the test medium, a larger inoculum (H=1.85) was taken. Here the increase in 8 days was only about 4.5-fold, though the cell population was still increasing rapidly between the 8th and 9th days. The growth rate depends somewhat on the inoculum size, and there is a maximum population that can be supported in a Carrel D-3.5 flask with 1 ml. of nutrient. H=10 is close to this maximum.

The effects on the growth of the cells of omitting (*a*) the protein, (*b*) the peptone, and (*c*) the ascorbic acid-cysteine-glutathione mixture are shown in text-figure 3. It is evident that, whereas omission of the




TEXT-FIGURE 1.—Growth of strain L cells in ML 192/2 over a period of 7 days. Small inoculum (H=0.55). Nutrient renewed at 2 and 4 days (*R*).



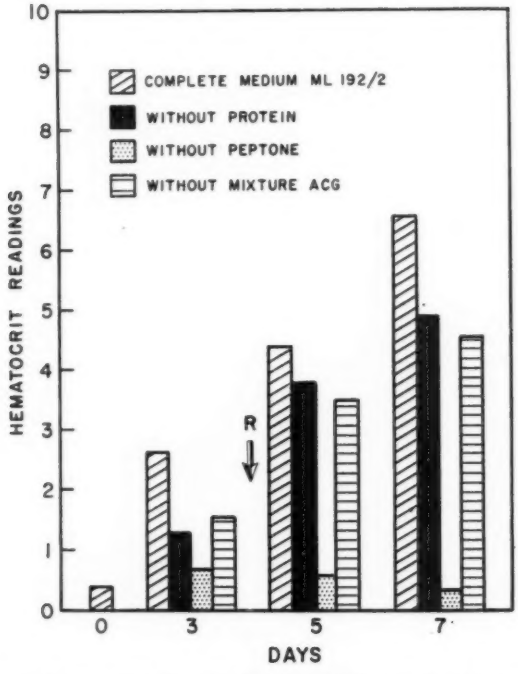

TEXT-FIGURE 2.—Growth of strain L cells in ML 192/2 over a period of 9 days. Large inoculum (H=1.8). Nutrient renewed at 3 and 7 days (*R*).

protein or of the reducing mixture only slightly decreases the growth, omission of peptone results in no proliferation.

To eliminate the possibility that the rapid growth of cultures removed for only a few weeks from a serum-containing nutrient might be due to some residual effect of the serum, cultures were also subjected to growth measurements after being maintained for several months in ML 192/2. A comparison of the growth of cultures which had been maintained for 14, 38, and 126 days, respectively, in ML 192/2 is shown in text-figure 4. After 18 weeks, it is clear that the cells were still proliferating at a similar, or even slightly higher, rate. There is no evidence of change in morphology or loss of vigor (fig. 2).

## Discussion

In the work described here, strain L mouse cells have been grown continuously for several months in a relatively simple nutrient solution containing, besides salts, sugars, amino acids, and water-soluble vitamins, 0.5 percent of Difco Bacto-Peptone and 0.05 percent of Armour's bovine albumin, fraction V. While neither the peptone nor the protein is a



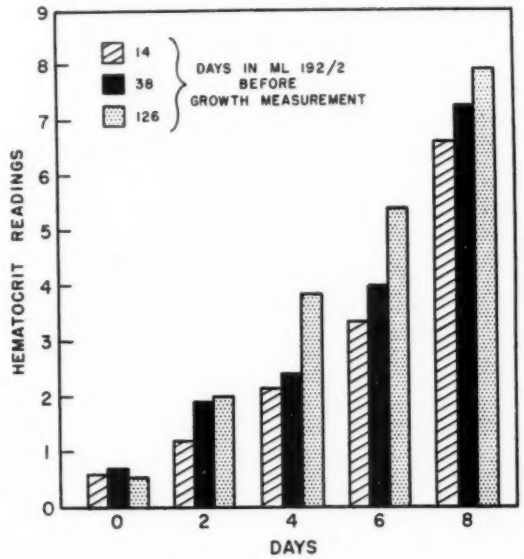

TEXT-FIGURE 3.—Comparison of growth of strain L cells in: (*a*) complete medium ML 192/2, (*b*) without protein, (*c*) without peptone, and (*d*) without mixture of ascorbic acid-cysteine-glutathione (ACG). Nutrient renewed at 4 days (*R*).

chemically defined component, each of these fractions contains predominantly high-molecular peptides or albumins, respectively, and only traces of other substances. These fractions are thus, although still complex, of a different order of complexity from serum or embryo extract, which each contain a wide range of biochemical components in physiologic concentrations. In the medium ML 192/2, no serum, embryo extract, or other materials of similar biological complexity were present. Both an amino acid mixture (used at about 8.6 mg. $NH_2$-N/100 ml.) and the peptone (at 75 mg. $NH_2$-N/100 ml.) were required to sustain rapid proliferation.

Some of the earliest reports on cell nutrition attest the value of peptones for promoting proliferation (*16–23*). Several of the first "artificial" media (*23–25*) contained peptones, usually Witte's peptone (a product of incomplete peptic digestion of fibrin). Willmer and Kendal (*26*) isolated a heteroproteose from Witte's peptone which, at 80 mg. N per 100 ml. and in the presence of plasma, was found to stimulate mitosis in cultures of chick-heart fibroblasts. Wilson, Jackson, and Brues (*27*) used a serum-free modification of Baker's (*24*) solution, and compared the growth of chick-embryo muscle in the presence of unmodified Witte's peptone (65 mg. $NH_2$-N/100 ml.) with that in a similar mixture containing

the peptone after complete hydrolysis to amino acids (equivalent to about 400 mg./100 ml.). Growth (proliferation) occurred in both media, though with marked morphologic differences between the cells in the two solutions. Unfortunately, Wilson *et al.* did not test the effect of a combination of unhydrolyzed peptone and amino acids. Hanks (*28*) found a rapid increase in cell population in cultures from chick-embryo muscle on adding Witte's peptone at 100 mg. per 100 ml. to a serum ultrafiltrate medium.

Our experience indicates that a peptone, in the presence of a mixture of amino acids and an otherwise synthetic nutrient solution, supplies an essential threshold factor for the growth of the strain L cells. The nature of the functional components in the peptone is under investigation. In spite of the suggestion made as far back as 1949 (*29*) that "We have to seek for the components possessing maximal activity in respect of the induction of cell proliferation between some special definite limits of molecular weight, namely, the upper limit of trichloroacetic acid precipitability and the lower limit of non-dialyzability, . . ." no specific fractions have been isolated from peptones, to which nutritional or growth-stimulating activities could be attributed, except the heteroproteose of Willmer and Kendal (*26*). Ehrensvärd (*30*) draws attention to the possibilities that "an inhibitory effect, caused by a temporary dominance of one amino acid, may be relieved by peptides; in other cases a continual destruction of an amino acid (by oxidative deamination, for




TEXT-FIGURE 4.—Comparison of growth of strain L cells in ML 192/2, maintained for (*a*) 14 days, (*b*) 38 days, and (*c*) 126 days before growth measurement. Nutrient renewed at 3 and 7 days (*a* and *c*) and at 4 and 7 days (*b*).

example) may be by-passed by the local administration of the same amino acid in peptide combination." It is by no means excluded that peptides themselves are assimilated by the cells. Winnick (*31,32*) has indicated the possibility that not only peptides but also even whole protein molecules may be utilized by cells in tissue culture.

In addition to 60 mg. per 100 ml. amino acids, the solution ML 192/2 contains 5.0 mg. per 100 ml. glutamine and 7.5 mg. per 100 ml. cysteine. Higher concentrations of amino acids cannot substitute for the peptone, nor can glutathione, which is included at 1.5 mg. per 100 ml. Dipeptides have not been tried (*cf. 33*). The quantities of amino acids used may be compared with the figures of 5 mg. per 100 ml. plus 7.3 mg. per 100 ml. glutamine, found by Eagle (*9,34*) to be optimum for this strain of cells in somewhat different conditions (*e.g.*, in the presence of dialyzed horse serum). Evans *et al.* (*35,36*) used about 42.2 mg. per 100 ml. amino acids in their media NCTC 107 and 108, that is about 75 percent of the amount in ML 192/2. Most of the other nutrients in common use, *e.g.*, Morgan, Morton, and Parker's 199 (*37*), Parker's 703 (*12*) and 858 (*13*), Morgan's M 150 (*38*), White's $W_H$ (*39*), and Trowell's TACPI (*40*), use concentrations 50 to 100 percent higher (92 to 136 mg./100 ml.) than in ML 192/2. It is of interest that Trowell, whose medium TACPI for rat lymphocytes contains 107 mg. per 100 ml. amino acids, found no favorable effect on survival from the addition of peptones or protein. But proliferation was not, in this case, the objective. It appears that media containing amino acids are sufficient for survival, but that the addition of other components present in the peptone can convert a nutrient suitable for maintenance of strain L cells into one which promotes their proliferation.

A protein fraction, Armour's bovine albumin, fraction V, has been included in ML 192/2. When peptone is present, proliferation at a rate only slightly less can occur in the absence of the protein; but the albumin fraction in the absence of peptone has no significant effect on proliferation. The action of the protein, like that of the peptone, is not fully understood. Either of these components may, for example, contain growth factors other than the protein or peptides themselves. Either or both may act in a buffering capacity or as a carrier or absorbent of some nutritionally important components. The probable unspecific nature of the protein's function is suggested by the finding of Sanford *et al.* (*6*) that all the protein fractions of horse serum tested were equally effective as supplements to their protein-free media for strain L cells. These authors, however, found bovine fractions ineffective in their experiments. This is, however, to be expected in a multivariable system; with one set of variables, one supplement may provide a missing factor, and with another set of variables, other completing factors may be required.

The mixture of reducing substances (ascorbic acid, cysteine, and glutathione) has appeared in most of the synthetic and semisynthetic media based on those of Vogelaar (*23*) and Baker (*24*). They are also found in the media 199 of Morgan, Morton, and Parker (*37*), 703 and 858 of

Parker (*12,13*), and NCTC 107 of Evans *et al.* (*35*). These substances were omitted from the synthetic maintenance media described earlier (*14*), chiefly because it was desired to avoid unstable components, and also because their effect upon prolonged survival was negligible. It was found, however, that inclusion of this mixture significantly improved the growth of strain L cells in media of the type described here (text-fig. 3). It is not the purpose of this paper to cite in detail the many experiments that led up to the final composition of medium ML 192/2, which include those upon which the retention of this group of substances is based. These will be reported elsewhere.

The medium ML 192/2, though it has been devised as a step toward a fully defined medium, and has so far only been used with strain L cells, may be found useful for investigations requiring cell proliferation in a serum-free environment. Experiments now in progress indicate that simple protein-free media able to support similar rates of growth will shortly become available.

## Summary

A nutrient solution is described, containing protein and a peptone but otherwise chemically defined. In this serum-free solution, rapid proliferation of strain L mouse cells is achieved, and can be maintained undiminished for several months.

## Bibliography

(*1*) Earle, W. R., Schilling, E. L., Stark, T. H., Straus, N. P., Brown, M. F., and Shelton, E.: Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(*2*) Sanford, K. K., Earle, W. R., and Likely, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1948.

(*3*) Likely, G. D., Sanford, K. K., and Earle, W. R.: Further studies on the proliferation *in vitro* of single isolated tissue cells. J. Nat. Cancer Inst. 13: 177–184, 1952.

(*4*) Sanford, K. K., Waltz, H. K., Shannon, J. E., Jr., Earle, W. R., and Evans, V. J.: The effects of ultrafiltrates and residues of horse serum and chick-embryo extract on proliferation of cells *in vitro*. J. Nat. Cancer Inst. 13: 121–137, 1952.

(*5*) Sanford, K. K., Leadbetter, P. A., Bryant, J. C., Braker, W. P., Evans, V. J., and Earle, W. R.: The effects of amino acids as supplements to fractions of serum and embryo extract on the proliferation of cells *in vitro*. J. Nat. Cancer Inst. 14: 513–518, 1953.

(*6*) Sanford, K. K., Westfall, B. B., Fioramonti, M. C., McQuilkin, W. T., Bryant, J. C., Peppers, E. V., Evans, V. J., and Earle, W. R.: The effect of serum fractions on the proliferation of strain L mouse cells *in vitro*. J. Nat. Cancer Inst. 16: 789–802, 1955.

(*7*) Westfall, B. B., Peppers, E. V., Sanford, K. K., and Earle, W. R.: The amino acid content of the ultrafiltrate from horse serum. J. Nat. Cancer Inst. 15: 27–35, 1954.

(*8*) Westfall, B. B., Peppers, E. V., and Earle, W. R.: The free and combined amino acids in the protein-free ultrafiltrates from whole chick-embryo extracts. J. Nat. Cancer Inst. 15: 433–438, 1954.

(*9*) Eagle, H.: The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. Biol. Chem. 214: 839–852, 1955.

(*10*) Evans, V. J., Bryant, J. C., Sanford, K. K., and Earle, W. R.: Studies of certain defined media for testing proliferation in replicate tissue cultures. Ann. New York Acad. Sc. 58: 1012–1020, 1954.

(*11*) Fioramonti, M. C., Bryant, J. C., McQuilkin, W. T., Evans, V. J., Sanford, K. K., and Earle, W. R.: The effect of horse serum residue and chemically defined supplements on proliferation of strain L clone 929 cells from the mouse. Cancer Res. 15: 763–766, 1955.

(*12*) Healy, G. M., Fisher, D. C., and Parker, R. C.: Nutrition of animal cells in tissue culture. IX. Synthetic medium No. 703. Canad. J. Biochem. & Physiol. 32: 327–337, 1954.

(*13*) ———: Nutrition of animal cells in tissue culture. X. Synthetic medium No. 858. Proc. Soc. Exper. Biol. & Med. 89: 71–77, 1955.

(*14*) Waymouth, C.: Simple nutrient solutions for animal cells. Texas Rep. Biol. & Med. 13: 522–536, 1955.

(*15*) ———: A rapid quantitative hematocrit method for measuring increase in cell population of strain L (Earle) cells cultivated in serum-free nutrient solutions. J. Nat. Cancer Inst. 17: 305–313, 1956.

(*16*) Smyth, H. F.: A new medium for the cultivation of chick tissues in vitro, with some additions to the technic. J. Med. Res. 31: 255–259, 1914.

(*17*) Kuczynski, M. H., Tenenbaum, E., and Werthemann, A.: Untersuchungen über Ernährung und Wachstum der Zellen erwachsener Säugetiere in Plasma unter Verwendung wohlcharakterisierter Zusätze an Stelle von Gewebsauszügen. (Nebst einem Anhang über den Nachweis der Immunkörperbildung seitens sprossender retikulärer Zellen in der Gewebskultur). Arch. path. Anat. 258: 687–718, 1925.

(*18*) Carrel, A., and Baker, L. E.: The chemical nature of substances required for cell multiplication. J. Exper. Med. 44: 503–521, 1926.

(*19*) ———: Chemical nature of some substances required for the growth of fibroblasts and epithelial cells. Proc. Soc. Exper. Biol. & Med. 23: 627–628, 1926.

(*20*) ———: Le rôle des produits de l'hydrolyse incomplète de la fibrine dans la prolifération cellulaire. Compt. rend. Soc. biol. 96: 685–687, 1927.

(*21*) Fischer, A., and Demuth, F.: Eiweissabbauprodukte als wachstumsfördernde Substanzen. Arch. exper. Zellforsch. 5: 131–142, 1927.

(*22*) Baker, L. E., and Carrel, A.: The effects of digests of pure proteins on cell proliferation. J. Exper. Med. 47: 353–370, 1928.

(*23*) Vogelaar, J. P. M., and Erlichman, E.: A feeding solution for cultures of human fibroblasts. Am. J. Cancer 18: 28–38, 1933.

(*24*) Baker, L. E.: Artificial media for the cultivation of fibroblasts, epithelial cells and monocytes. Science 83: 605–606, 1936.

(*25*) Baker, L. E., and Ebeling, A. H.: Artificial maintenance media for cell and organ cultivation. I. The cultivation of fibroblasts in artificial and serumless media. J. Exper. Med. 69: 365–378, 1939.

(*26*) Willmer, E. N., and Kendal, L. P.: The utilization of proteoses by chicken heart fibroblasts growing *in vitro*. J. Exper. Biol. 9: 149–179, 1932.

(*27*) Wilson, H., Jackson, E. B., and Brues, A. M.: The metabolism of tissue cultures. I. Preliminary studies on chick embryo. J. Gen. Physiol. 25: 689–703, 1942.

(*28*) Hanks, J. H.: The longevity of chick tissue cultures without renewal of medium. J. Cell. & Comp. Physiol. 31: 235–260, 1948.

(*29*) Ehrensvärd, G., Fischer, A., and Stjernholm, R.: Protein metabolism of tissue cells *in vitro*. 7. The chemical nature of some obligate factors of tissue cell nutrition. Acta physiol. scandinav. 18: 218–230, 1949.

(*30*) Ehrensvärd, G.: Metabolism of amino acids and proteins. Ann. Rev. Biochem. 24: 275–310, 1955.

(*31*) Winnick, T.: Tissue culture methods in relation to biochemical studies. Texas Rep. Biol. & Med. 1: 452–462, 1952.

(*32*) Winnick, R. E., and Winnick, T.: Utilization of amino acids, peptides, and

protein by cultures of embryonic heart. J. Nat. Cancer Inst. 14: 519–525. 1953.

(*33*) EAGLE, H.: Utilization of dipeptides by mammalian cells in tissue culture. Proc. Soc. Exper. Biol. & Med. 89: 96–99, 1955.

(*34*) ———: Nutrition needs of mammalian cells in tissue culture. Science 122: 501–504, 1955.

(*35*) EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. I. A protein-free chemically defined medium for cultivation of strain L cells. Cancer Res. 16: 77–86, 1956.

(*36*) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. 16: 87–94, 1956.

(*37*) MORGAN, J. F., MORTON, H. J., and PARKER, R. C.: Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exper. Biol. & Med. 73: 1–8, 1950.

(*38*) PASIEKA, A. E., MORTON, H. J., and MORGAN, J. F.: The metabolism of animal tissues cultivated *in vitro*. I. Amino acid metabolism of chick embryonic-heart fibroblasts cultivated in synthetic medium M 150. J. Nat. Cancer Inst. 16: 995–1009, 1956.

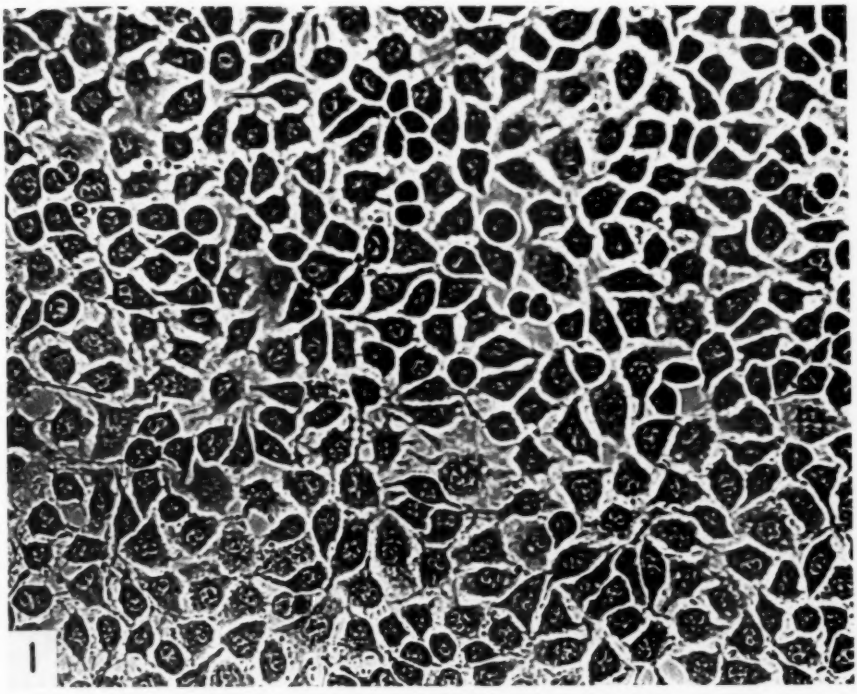
(*39*) WHITE, P. R.: An approach to chemically defined nutrients for strain L mouse cells. J. Nat. Cancer Inst. 16: 769–787, 1955.

(*40*) TROWELL, O. A.: The culture of lymph nodes in synthetic media. Exper. Cell Res. 9: 258–276, 1955.

PLATE 20

FIGURE 1.—Six-day-old stock culture of strain L cells grown for 435 days in ML 97. × 240

FIGURE 2.—Six-day-old stock culture of strain L cells grown for 126 days in ML 192/2. × 240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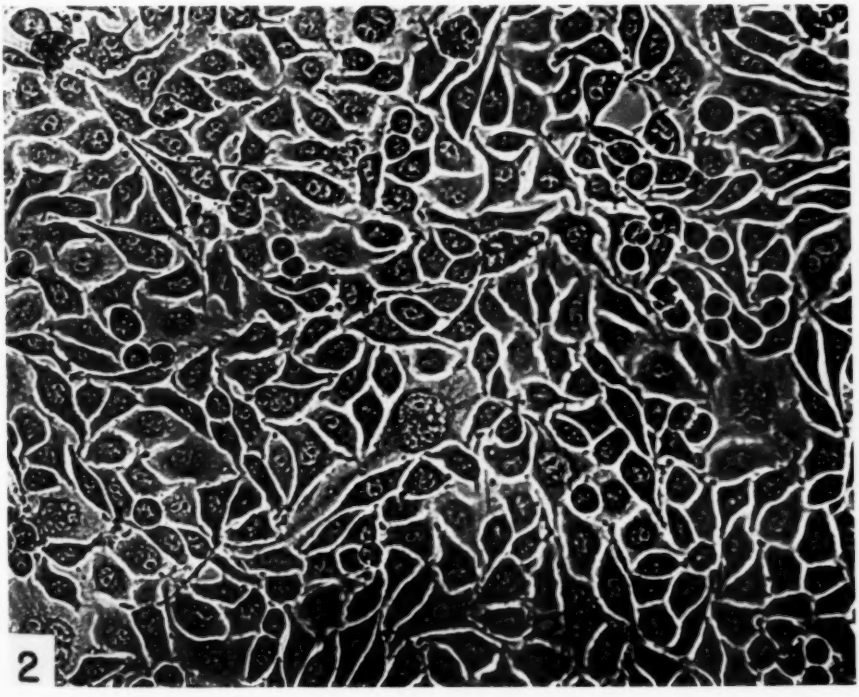
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# Pathologic Desquamation of Oral Epithelium Examined by Electron Microscopy and Histochemistry [1, 2]

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This study is centered around a case report on desquamative stomatitis. Biopsy specimens have been examined by certain methods which have not been previously applied to a similar problem. We believe, therefore, that the observations may have some general and fundamental interest, especially with respect to the cornification and desquamation processes.

## Case History and Physical Findings

The case in question is that of a 71-year-old female who first reported to our clinic 13 years ago at the age of 58. Her chief oral complaint at that time was persistent redness and bleeding of the oral mucosa; her chief general complaint was hot flashes. The principal physical findings were reddened areas on the mucosa of the palate adjacent to the maxillary left and right molar regions, diffuse gingivitis involving the mandibular anterior teeth, and similar inflammatory involvement of the buccal mucosa of the left cheek. The history and clinical findings were compatible with a diagnosis of desquamative stomatitis.

In view of a possible relationship between this condition and postmenopausal hormonal status, an attempt was first made to improve the oral condition by administration of stilbestrol. During 8 months' treatment with this hormone, the hot flashes were controlled, but the oral mucous-membrane lesions persisted. In the hope of controlling the tendency to infection accompanying the inflamed lesions, sulfanilamide was administered, but again with no obvious effect on the reddened areas.

By this time the involvement adjacent to the mandibular anterior teeth and the maxillary molars was so extensive that a surgical approach was decided upon. Following an exploratory gingivectomy on the left side of the lower anterior region and extraction of the upper left molars, primary healing did take place. However, this initially encouraging result was

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shortly followed by recurrence of the raw and red inflamed soft-tissue lesions in the same regions. During the late 1940's the new antibiotics, penicillin and aureomycin, were administered with a view to controlling the infection, but again with negative results.

The nutritional status of the patient appeared satisfactory. Therapeutic supplements of specific vitamins—nicotinic acid, thiamin, riboflavine, vitamin A, yeast tablets, and crude liver extracts—failed to improve the condition. In 1955, treatment with cortisone was tried: first, topical application of cortisone ointment to the mucosa 3 times a day, with no effect; second, oral administration of cortisone, 50 mg. a day for 2 months, with no effect; and third, oral administration of metacorten, 5 mg. a day for 3 months, again with no effect.

We have examined the nature of the lesions—which have persisted for a decade—by a variety of laboratory methods: smears from the mucous membrane stained by the Papanicolaou method (*1*); biopsies examined by various histologic and histochemical techniques referred to below;[3] and finally, thin sections of osmium-fixed, butyl methacrylate-embedded specimens studied by electron microscopy, using the RCA EMU–3 instrument at 50 and 100 KV, and principally intermediary magnifications.

## Histologic and Histochemical Observations

It became evident both from the oral smears and the histologic sections that this patient had a tendency to desquamate squamous epithelial cells which had failed to reach a normal stage of maturity. Neither the smears (fig. 1) nor the sections (fig. 2) exhibited the typical large, peripheral, pyknotic cells with pale fibrous cytoplasm normally encountered in mature squamous cells of the oral mucosa.

By histochemical means we have examined the relative distribution of protein-bound sulfhydryl groups and disulfide bonds, using the methods of Barrnett and Seligman (*2*) Tissues so examined revealed the presence of sulfhydryl groups throughout the epithelial layer, with a considerable and unexplained concentration of this reaction in the superficial cells (fig. 3). However, the disulfide-bond reaction was essentially negative throughout the epithelium, since tests for this component coupled with the sulfhydryl reaction gave little or no additional activity (fig. 4). Glycogen was not materially reduced in the stratum spinosum beyond normal control material. Basophilic reactions were quite marked in the nuclei, and the cytoplasm was also stained quite deeply. Alkaline-phosphatase reaction was principally concentrated along the walls of the blood vessels in the underlying connective tissues. With the toluidine-blue method, following basic lead-acetate fixation, the amorphous ground substance was poorly stained, but there was nevertheless an abundance of mast cells as revealed by the intense metachromasia of their cytoplasmic granules.

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[3] The various fixatives and other chemical reagents used in the histochemical tests have been described in detail in previous work (Wislocki, G. B., and Sognnaes, R. F.: Am. J. Anat. 87: 239–276, 1950; and Sognnaes, R. F., and Lustig, L.: J. Dent. Res. 34: 132–143, 1955) dealing with the normal tissues of the mouth.

## Electron-Microscopic Findings

Our observations with the electron microscope have so far been concentrated on the epithelial tissue. In a related electron-microscopic study we have noted that the normal keratinization process is accompanied by distinct nuclear and nucleolar changes which appear to be related to the formation of keratohyalin granules and keratogenesis (*3*).

In the tissues from desquamative stomatitis, on the other hand, the nuclei appeared to remain round and dense. The principal alterations seemed to occur in the peripheral cytoplasm and the intercellular regions of the epithelial cells. Even the cells adjacent to the basement membrane showed what appeared to be disintegration of the intercellular "bridge" material and destruction of the attachment between the peripheral cytoplasm and the cell membranes (figs. 5 and 6). Extravascular blood cells could be found penetrating between the poorly attached epithelial cells (fig. 7).

Progressing toward the external surface layer of the epithelium, there was a surprisingly good preservation of large, round nuclei which appeared to be in normal relationship to the adjacent cytoplasm, within which there was also noted an abundance of mitochondria but an absence of keratohyalin granules (fig. 8).

In the external cell layers the peripheral cytoplasm and the cell membranes appeared to become increasingly disoriented and separated. Near the oral surface one could find detached cells, presumably corresponding to those seen in the oral smears (fig. 1), which, from the point of view of size, shape, and appearance of the nuclei, were essentially of a basal-cell nature (figs. 8 and 9). Furthermore, the foldings and interdigitations previously described in the region between the normal epithelial-cell surfaces of the mucous membranes were not present (*3*). There was little evidence of fibrillar elements in the perinuclear cytoplasm. Keratohyalin granules were absent, whereas mitochondrial bodies were present in the cytoplasm even in the superficial desquamating cells (fig. 9).

## Discussion

Aside from a preliminary report of our own (*3*), we are not aware of any previous electron-microscopic observations on the squamous epithelium in either normal or pathologic mucous membrane of the mouth. Previous histochemical observations in desquamative gingivitis have been directed principally toward the nature of the underlying connective tissue (*4*).

Our histologic, histochemical, and electron-microscopic observations suggest that one of the important changes in desquamative stomatitis is the failure to produce keratin fibrils within the cytoplasm of the squamous epithelial cells. We have noted that the precursors for keratin formation were present. The sulfhydryl-bound protein appeared to be normal. The nuclear and nucleolar core of the epithelial cells appeared morphologically intact. Even in the superficial desquamating cells mito-

chondria were abundant. It would seem, therefore, that one of the important mechanisms of this disease is the involvement of the epithelium indicated by: failure to develop keratohyalin granules, deficient formation of disulfide bonds, lack of elaboration of fibrous protein in the peripheral cytoplasm, sparsity of intercellular amorphous substance, and absence of interdigitation of the cell membranes of the superficial squamous cells.

The fact that this case has been extremely resistant to hormonal, nutritional, antibiotic, and surgical treatment suggests that either a combination of these factors or some other specific factor, presumably related to the keratinization process, is involved in the disease process.

From a structural point of view, there is still considerable confusion regarding the elaboration of the intracellular keratin fibrils in squamous epithelial cells. The possible involvement of the keratohyalin granules in this process has received considerable attention ever since Waldeyer (*5*) described these granules and attributed their presence to degeneration of the epithelial cells. Although the subject is still debatable, in recent literature quite categorical statements have appeared about the origin and composition of the keratohyalin granules. Because of their intense basophilia, they have been thought to originate from chromatin material and to contain deoxyribonucleoprotein (*6*).

Our preliminary observations with the electron microscope suggest the possibility that the nucleoli of the squamous epithelial cells may contribute to the formation of these granules (*3*). In this connection it is interesting to note that Caspersson (*7*), using the ultraviolet absorption method, found a great accumulation of cytoplasmic ribonucleotides during the keratinization process. Similarly, Leuchtenberger and Lund (*8*) compared the amount of deoxyribonucleic acid by means of the Feulgen reaction in the malpighian layer and the stratum granulosum of epidermal cells without finding any significant difference. Taken together, these two studies would seem to suggest that during the time of keratinization when the keratohyalin granules are abundant, there is a concentration of ribonucleoprotein rather than deoxyribonucleoprotein in the cytoplasm. The suggestion has been made, therefore, that the keratohyalin granules might arise from the cytoplasm rather than the nucleoplasm (*8*). At the time of this writing, a report by Sheldon and Zetterquist (*9*)—based on the basis of electron-microscopic examination of the cornea of vitamin A-deficient mice—has implicated the mitochondria in keratohyalin formation.

Our electron-microscopic findings do not rule out that both the cytoplasm and the nucleoplasm may contribute to the elaboration and chemistry of these granules. But we have been particularly impressed by the changes in the nucleoli (*3*). At the stage of keratinization the nucleoli appear to produce satellites which migrate to the nuclear membrane, where electron-dense material concentrates and appears to be expelled into the adjacent cytoplasm. It is noteworthy that this nucleolar contribution to the granules still would be consistent with a high ribo-

nucleotide content of keratohyalin formed in this manner. This concept of nucleolar contribution to keratohyalin and keratin formation seems at the moment more compatible with our electron-microscopic findings than the traditional one claiming origin from chromatin material of the nucleoplasm (*6*). We are currently exploring this concept in greater detail.

Regardless of origin, it seems evident both from our histochemical and electron-microscopic findings that the keratinization process is one involving high metabolic activity, a result of growth rather than death of the tissue. Conversely, we have noted in conditions resulting in the piling up of keratin, namely in leukoplakia, that there appears to be a slowing down of tissue metabolism. In leukoplakia the sulfhydryl-group reactions appear to be reduced and the cells fail to shed, in contrast to desquamative stomatitis where the formation of the sulfhydryl precursors is high, the disulfide-bond reaction is low, and the cells are prematurely shed. In the latter situation we failed to improve the condition by hormone administration. In leukoplakia, on the other hand, Nathanson and Weisberger (*10*) have reported improvement following treatment with estrogenic hormone—possibly by reactivating formation of the precursors required for normal keratinization and desquamation. We are currently exploring further the ultrastructure and histochemical changes accompanying leukoplakia and other precancerous lesions of the oral mucosa.

## Summary

Oral smears and histologic, histochemical, and electron-microscopic sections of specimens from the oral mucosa of a 71-year-old woman who suffered from desquamative stomatitis have been examined and have proved to be extremely resistant to hormonal, nutritional, antibiotic, and surgical treatment over a period of more than 10 years.

From a microscopic point of view, the principal characteristic of the case was the persistence of immature epithelial cells of a basal-cell nature throughout the thickness of the epithelium. Such cells were apparent in the smears and in the histologic, histochemical, and electron-microscopic specimens. Histochemical reactions for sulfhydryl groups were quite pronounced, but there was an absence of disulfide-bond reactions in the peripheral epithelial cells. Throughout the epithelial layers the cell nuclei and nucleoli remained uniformly round and dense. Similarly, there was an abundance of mitochondrial bodies in the cytoplasm of the epithelial-cell layers, even in the peripheral ones.

However, there appeared to be a breakdown of the peripheral cytoplasm and a separation between the cell membranes and the cell proper, as well as between individual cells. Extravascular blood cells from the underlying connective tissue could be found invading these regions.

Near the oral surface the epithelial cells appeared to come apart readily as a result of a disintegration of the bond between the cell membranes; concurrently a separation of the cell membrane from the peripheral

cytoplasm was observed. The deep interdigitations between the cell surfaces seen in normal oral epithelium were not present. Keratohyalin granules and intracellular fibrils were sparse or absent, whereas the nuclear, nucleolar, and mitochondrial structures were retained relatively unchanged even in the peripheral cell layers.

## References

(*1*) PAPANICOLAOU, G. N.: Sexual cycle in the human female as revealed by vaginal smears. Am. J. Anat. (Supp.) 52: 519–637, 1933.

(*2*) BARRNETT, R. J., and SELIGMAN, A. M.: Histochemical demonstration of protein-bound sulfhydryl groups. Science 116: 323–327, 1952.

(*3*) SOGNNAES, R. F., and ALBRIGHT, J. T.: Preliminary observations on the fine structure of the oral mucosa. Anat. Rec. (in press), 1956.

(*4*) ENGEL, M. B., RAY, H. G., and ORBAN, B.: The pathogenesis of desquamative gingivitis, a disturbance of the connective tissue ground substance. J. Dent. Res. 29: 410–418, 1950.

(*5*) WALDEYER: Untersuchungen über die Histogenese der Horngebilde, insbesondere der Haare und Federn. *In* Beiträge zur Anatomie und Embryologie als Festgabe Jacob Henle. Bonn, Max Cohen & Sohn, 1882.

(*6*) ALLEN, A. C.: The Skin—A Clinicopathologic Treatise. St. Louis, C. V. Mosby Co., 1954.

(*7*) CASPERSSON, T. O.: Cell Growth and Cell Function. New York, W. Norton Company, 1950.

(*8*) LEUCHTENBERGER, C., and LUND, H. Z.: The chemical nature of the so-called keratohyaline granules of the stratum granulosum of the skin. Exper. Cell Res. 2: 150–152, 1951.

(*9*) SHELDON, H., and ZETTERQUIST, H.: Experimentally induced changes in mitochondrial morphology: Vitamin A deficiency. Exper. Cell Res. 10: 225–228, 1956.

(*10*) NATHANSON, I. T., and WEISBERGER, D. B.: Treatment of leukoplakia buccalis and related lesions with estrogenic hormone. New England J. Med. 221: 556–560, 1939.

# PLATES

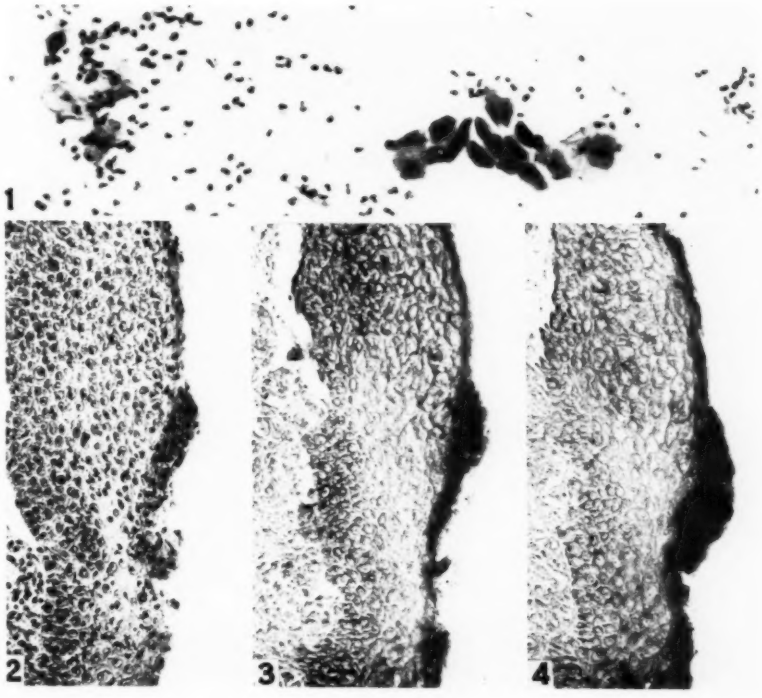
## PLATE 21

FIGURE 1.—Smear prepared from scraping of oral mucosa of patient suffering from desquamative stomatitis. *Note* the cluster of desquamated basal cells with large nuclei and dense basophilic cytoplasm. Papanicolaou's stain. × 40

FIGURE 2.—Biopsy from oral mucosa showing desquamative stomatitis. Oblique section through squamous epithelium. *Note* basal-cell nature of the superficial desquamating cells. Hematoxylin and eosin. × 175

FIGURE 3.—Section comparable to that in figure 2 stained for sulfhydryl-bound protein and reacting strongly in superficial cells. × 175

FIGURE 4.—Same as figure 3, stained for both sulfhydryl groups and disulfide bonds suggesting, by comparison, relatively little conversion to disulfide bonds. × 175




Sognnaes *et al.*



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PLATE 22

FIGURE 5.—Basement membrane and basal cells of oral mucosa affected by desquamative stomatitis. *Note* the dense, round epithelial nuclei and the relative abundance of mitochondria, whereas the peripheral cytoplasm and intercellular relationships are markedly distorted. × 13,000

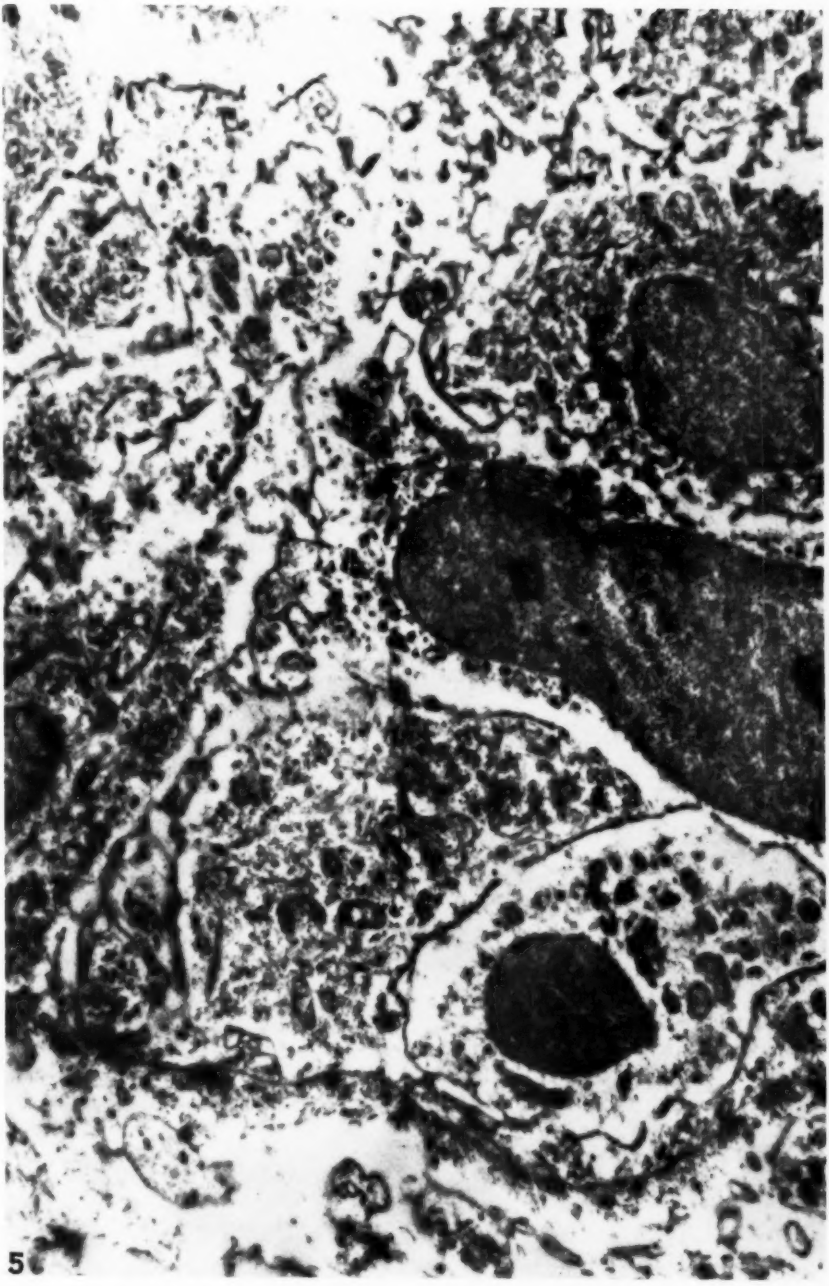
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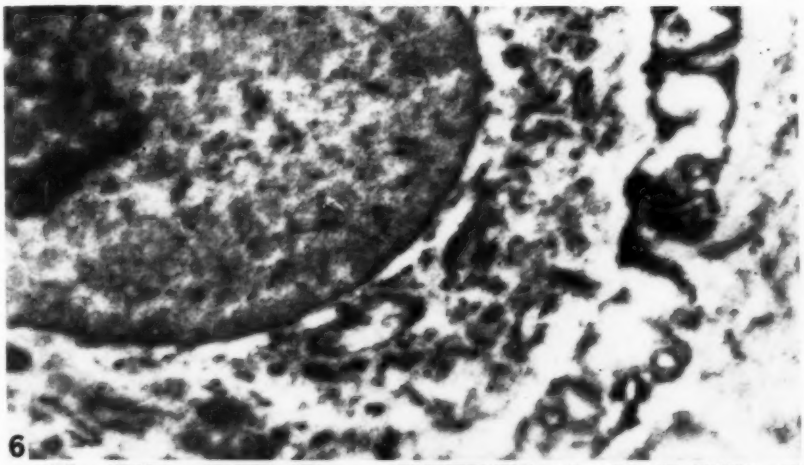
PLATE 23

FIGURE 6.—Premature separation of squamous cells. *Note* the dense nucleus (*left*), uniform nucleoplasm and nuclear membrane, and abundance of mitochondria, but distortion of relationship between peripheral cytoplasm and intercellular "bridge" material. × 35,000

FIGURE 7.—Portion of extravascular blood cell (eosinophil) penetrating between inadequately connected basal cells of oral epithelium. *Note* the electron-dense round nucleoli and nuclei and abundance of mitochondria compared to the deficient intercellular relationship. × 12,000



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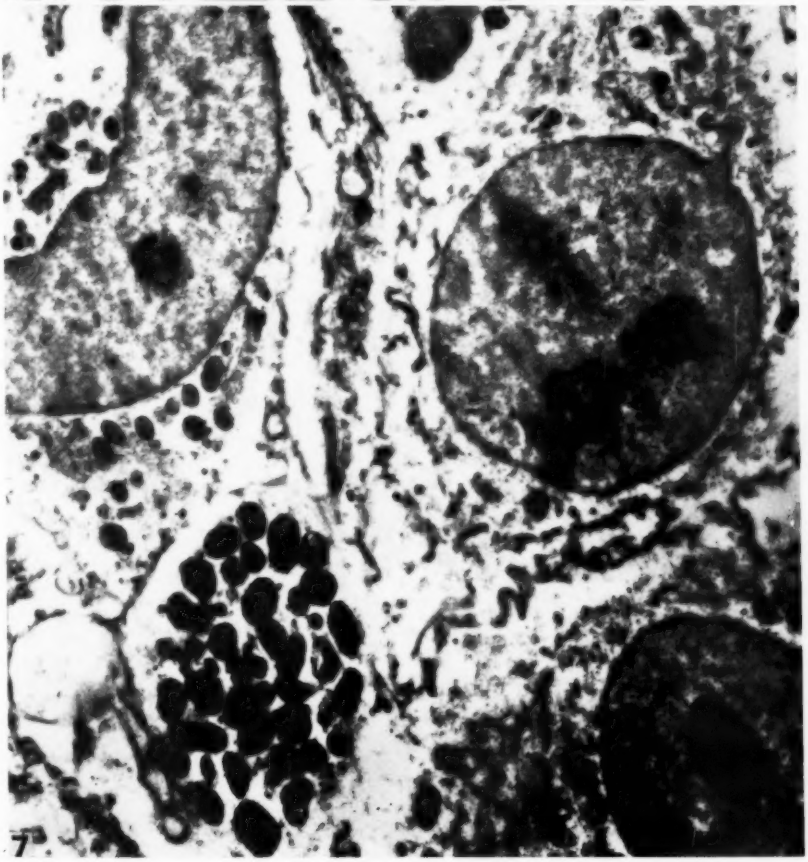

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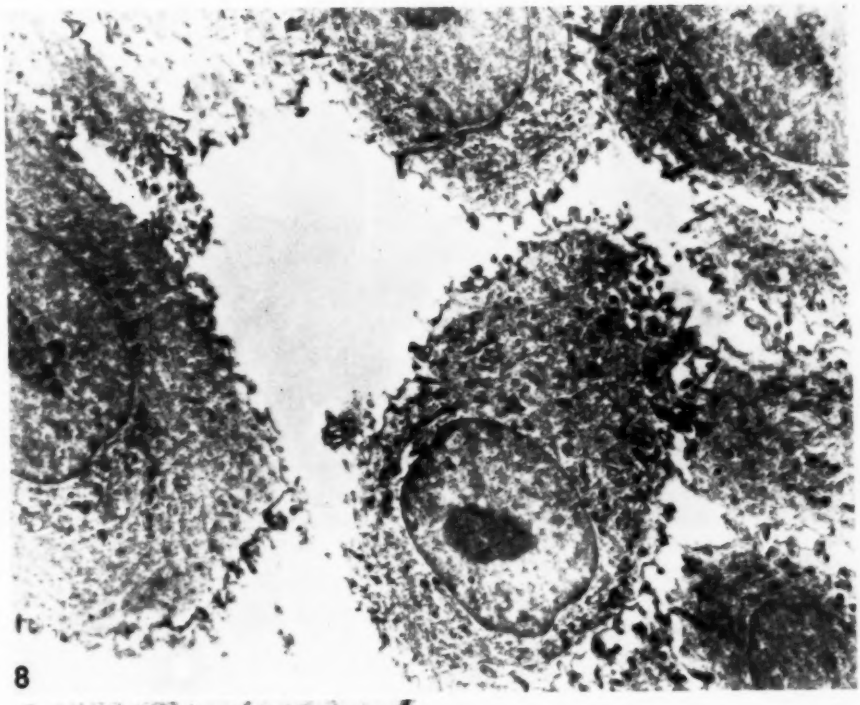
PLATE 24

FIGURE 8.—Abnormal separation of surface epithelial cells. *Note* retention of nuclei of basal-cell character and separation of cell membranes from peripheral cytoplasm and from each other. × 10,000

FIGURE 9.—Peripheral squamous cell of oral epithelium in desquamative stomatitis. *Note* the basal-cell characteristics of the nucleus and nucleolus, and absence of keratohyalin granules. × 19,000

FIGURE 10.—Completely isolated squamous cell, prematurely shed, comparable to cell within the smear shown in figure 1. × 11,000



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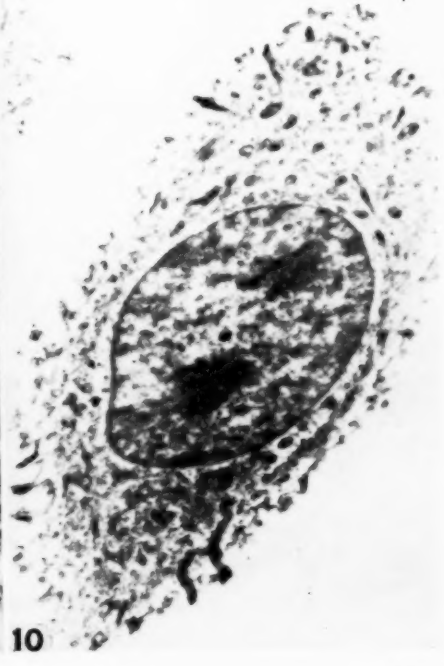

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# Urinary Metabolites of the Carcinogen *N*-2-Fluorenylacetamide [1, 2, 3]

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## Introduction

The studies on chemical carcinogenesis carried out in this laboratory were first devoted to developing sensitive methods (*1,2*) suited to the estimation of the compounds formed in the rat after ingestion of carcinogenic levels of *N*-2-fluorenylacetamide. It seemed important to investigate the metabolism of this compound in order to gain an understanding of the mechanism of its carcinogenic action. The initial experiments showed that *N*-2-fluorenylacetamide was converted rapidly to then unidentified substances (*3,4*) and that the distribution and excretion of *N*-2-fluorenylacetamide or its diazotizable metabolites was influenced by intake (*4*). These early studies proved quite inadequate to account for the many metabolic products of *N*-2-fluorenylacetamide produced by the rat and the use of isotopically labeled compounds proved necessary.

Following the administration of labeled *N*-2-fluorenylacetamide about two thirds of a single dose was excreted in the urine and the other third eliminated in the feces (*5–7*). Partition experiments revealed that a sizable amount of the chemical was metabolized into more polar, water-soluble products. Paper-chromatographic experiments confirmed these observations and indicated the existence of 12 or more urinary metabolites (*8*). Furthermore, radioactivity was bound to proteins of various rat tissues, especially the liver (*9,10*). After hydrolysis of the protein the distribution of radioactivity between phases of immiscible solvents was similar to that found in the urine. Therefore, investigation of the urinary metabolites was carried out first because such metabolites were more accessible and comprised a larger portion of a given dose of *N*-2-

[1] Received for publication May 21, 1956.

[2] Presented in part at the meetings of the American Society of Biological Chemists, Chicago, 1953 (Fed. Proc. 12: 287, 1953), the American Association for Cancer Research, Atlantic City, 1954 (Proc. Am. Assoc. Cancer Res. 1: 51, 1954), and the American Chemical Society, Cincinnati, Spring 1955 (Abstracts of papers, page 7C).

[3] The nomenclature of *Chemical Abstracts* for this compound will be used henceforth instead of the former designation, 2-acetylaminofluorene.

[4] We wish to acknowledge the competent technical assistance rendered by Mrs. Anna R. Parker during these investigations.

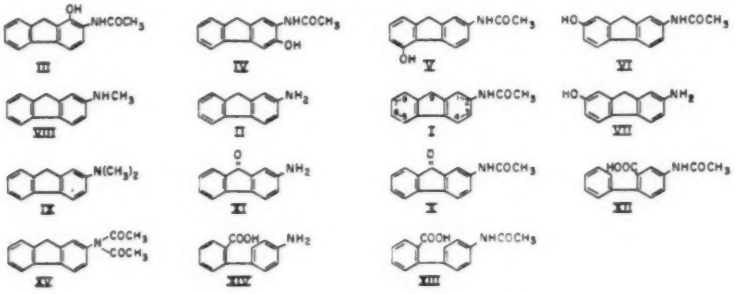
[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

fluorenylacetamide. The experience and knowledge thus acquired might facilitate determination of the structure of the protein-bound compounds in the tissues at a later time.

One approach to the elucidation of the various metabolites formed in the rat following the ingestion of *N*-2-fluorenylacetamide has been based mainly on the application of the carrier-isotope dilution technique (*11*). The carrier-isotope procedure involves the addition of a known non-radioactive compound (the carrier) to a solution of the radioactive unknown. Any compound in the solution chemically identical with the carrier will be inseparable when the latter is subsequently recovered and exhaustively purified.

This report presents the results obtained by the application of the aforementioned procedure to the determination of a number of urinary metabolites following a single dose of *N*-2-fluoren-9-$C^{14}$-ylacetamide in the rat.

*Some possible urinary metabolites of* N-*2-fluorenylacetamide.*—The chromatographic separation of at least 12 compounds from the urine of rats fed *N*-2-fluorenylacetamide necessitates a consideration of the possible metabolites formed. The substances illustrated in text-figure 1



TEXT-FIGURE 1.—Some of the possible metabolites of *N-2*-fluorenylacetamide.

I. *N*-2-Fluorenylacetamide (2-acetylaminofluorene—name previously used given in parentheses where different from usage in *Chemical Abstracts*)
II. 2-Fluorenamine (2-aminofluorene)
III. *N*-(1-Hydroxy-2-fluorenyl)acetamide (2-acetylamino-1-hydroxyfluorene)
IV. *N*-(3-Hydroxy-2-fluorenyl)acetamide (2-acetylamino-3-hydroxyfluorene)
V. *N*-(5-Hydroxy-2-fluorenyl)acetamide (2-acetylamino-5-hydroxyfluorene)
VI. *N*-(7-Hydroxy-2-fluorenyl)acetamide (2-acetylamino-7-hydroxyfluorene)
VII. 7-Amino-2-fluorenol* (2-amino-7-hydroxyfluorene)
VIII. *N*-Methyl-2-fluorenamine (2-methylaminofluorene)
IX. *N,N*-Dimethyl-2-fluorenamine (2-dimethylaminofluorene)
X. *N*-(9-Oxo-2-fluorenyl)acetamide (2-acetylaminofluorenone)
XI. 2-Amino-9-fluorenone (2-aminofluorenone)
XII. 4-Acetamido-2-biphenylcarboxylic acid
XIII. 4′-Acetamido-2-biphenylcarboxylic acid
XIV. 4′-Amino-2-biphenylcarboxylic acid
XV. *N*-2-Fluorenyldiacetamide (2-diacetylaminofluorene)

*Data for the various isomeric aminofluorenols will be reported in a subsequent paper.

might be some of these metabolites. One step which might be expected in the metabolism of *N*-2-fluorenylacetamide would be deacetylation leading to 2-fluorenamine. This reaction has been shown to take place both *in vivo* (*5*) and *in vitro* (*12,13*). However, Dyer (*14*) has found only a small amount of free diazotizable material, and Gutmann and Peters (*15*) reported the presence of only a trace of 2-fluorenamine in the urine of rats fed *N*-2-fluorenylacetamide.

*N*-Methylation of aromatic amines (*10,16*) and *N*-diacetylation (*12*) are still other possible metabolic pathways. *N*-Methyl-2-fluorenamine, *N,N*-dimethyl-2-fluorenamine, and *N*-2-fluorenyldiacetamide would be obtained if these reactions occurred during the metabolism of *N*-2-fluorenylacetamide. Unless such compounds were metabolized further, some portion could appear in the urine.

Hydroxylation is another important metabolic pathway of aromatic ring systems (*17*). Bielschowsky (*18*) first isolated a small amount of *N*-(7-hydroxy-2-fluorenyl)acetamide from the urine of rats fed *N*-2-fluorenylacetamide, but no quantitative estimate of the relative importance of this metabolite could be made then. Theoretical considerations (*19*) indicated that of all the possible 2-acetamido-x-hydroxyfluorenes, the 1-, 3-, 5-, and 7-hydroxylated derivatives could be metabolites of *N*-2-fluorenylacetamide. In view of the known conjugation of phenolic compounds with sulfuric or glucuronic acid (*20,21*), these derivatives in turn might be expected to occur. Moreover, deacetylation of the 2-acetamido-x-hydroxyfluorenes would produce the corresponding 2-amino-x-hydroxy derivatives as well as their conjugates. The latter compounds could also result, of course, from the hydroxylation and conjugation of 2-fluorenamine.

Quinones have been identified as the metabolic oxidation products of certain polynuclear aromatic hydrocarbons (*22*). The quinones underwent further reactions involving the splitting of the bond between the carbonyl and adjacent carbon with production of a carboxylic acid (*23*). The 9-carbon in fluorene is of a somewhat more aliphatic type than the carbons in the aforementioned hydrocarbons, but it is conceivable that similar reactions might occur. Thus *N*-(9-oxo-2-fluorenyl)acetamide, 2-amino-9-fluorenone, and, by ring opening, 4-acetamido-2-biphenylcarboxylic acid or the isomeric 4′-acetamido-2-biphenylcarboxylic acid and the corresponding amines could be metabolites of *N*-2-fluorenylacetamide.

## Methods and Results

*Care of animals.*—Three- to 6-month-old female Buffalo strain rats, weighing 130 to 190 gm., were given, by stomach tube, a single dose of the carcinogen *N*-2-fluoren-9-$C^{14}$-ylacetamide at the dose of 10 mg. of compound per 100 gm. of body weight. This amount of *N*-2-fluorenylacetamide on repeated weekly feeding was found to induce tumors (*24*). The animals were placed in glass metabolism cages permitting the separate collection of urine and feces. The urine was collected in a vessel cooled

by an ice bath. Food and water were available *ad libitum*. Our previous experiments (*5*) had demonstrated that a substantial portion of the urinary radioactivity was excreted in the first 48 hours following the feeding of the labeled compound. Therefore the pooled 0- to 48-hour fraction of urine, simply referred to as "urine," was used in the isotope-dilution experiments.

*Radioactivity determinations.*—The samples were counted at each step in the various carrier-isotope dilution experiments. In the early stages of the purification procedures the filter cake (about 20 to 22 mm. in diameter) was counted with an end-window Geiger-Müller tube (*25*).

In later stages of the process the specific radioactivity (s.a.) of the samples was determined and expressed in counts per minute per millimole (c.p.m. per m*M*). For this purpose a known weight of about 10 mg. of sample dissolved in the appropriate solvent was made up to a volume of 1 ml. Almost infinitely thin layers of the material were then spread over an area of 11.5 cm.$^2$ by pipetting a 25 to 50 $\mu$l. aliquot on 1.5-inch diameter glass disks. Such plates were always prepared and counted in duplicate or triplicate in a gas-flow windowless counter, with a probable error of 2 to 3 percent except on samples of very low activity. Such materials were counted for at least 1 hour in a Robinson-type (*26*) counter, which had a background-counting rate of 3.8 c.p.m.; in the final stages of an experiment a sample count of at least 8 hours was sandwiched between 2 background counts of similar duration to give data of reasonable reliability. This was especially important when a compound was not a metabolite and counted at or close to background rates. At appropriate stages the melting points of the materials were taken on a Kofler block.

*Use of specific adsorbents.*—In the course of preliminary experiments it was found that the efficiency of purification of the carrier compound was increased appreciably by the judicious use of adsorbents either in a column or in a batch process. For example, Norit adsorbed almost completely and practically irreversibly the ether-soluble metabolites of *N*-2-fluorenylacetamide, as shown in table 1. This was also demonstrated in the case of the $N^{15}$-labeled carcinogen (*27*). In the instance cited the adsorbent was 1,000-fold in excess of the adsorbed metabolites; hence

TABLE 1.—*Adsorption of ether-soluble metabolites of* N*-2-fluorenylacetamide on Norit**

| Fraction | Counts recovered | Percent recovered |
|---|---|---|
| Ether | 59 | 0. 36 |
| Ether-ethanol, 1:1 | 78 | 0. 47 |
| Ethanol | 80 | 0. 49 |
| Ethanol-acetic acid, 1:1 | 130 | 0. 79 |
| Acetic acid | 110 | 0. 67 |
| Ethanol-ammonia in Soxhlet for 20 hrs | 608 | 3. 7 |
| Total recovered | 1, 065 | 6. 5 |

*An ether extract of urine containing 16,400 c.p.m., equivalent to 26 $\mu$g. *N*-2-fluorenylacetamide, was added to 46 mg. of Norit and the activity was eluted by solvents of increasing polarity, as shown. For the last step the Norit was transferred to a micro-Soxhlet extractor.

removal from the solution was complete. In our experiments an amount of Norit equivalent to 5 to 20 percent of the weight of the carrier compound was generally employed. Under those conditions a competitive equilibrium between the various solutes for the adsorbent surface existed, so that in favorable cases most of the contaminants were adsorbed. Columns of alumina acted similarly except that the adsorptive capacity was more selective. Polar compounds such as phenols were adsorbed from benzene solution and could be eluted by ethanol, while 2-fluorenamine and *N*-substituted basic or neutral derivatives were not adsorbed from benzene. Advantage was taken of these properties in the following experiments.

*General procedure.*—The metabolites from a labeled precursor have, of course, the same specific molar radioactivity as the parent compound provided that no dilution from endogenous sources arises, which is evidently impossible in the case of the synthetic carcinogen *N*-2-fluoren-9-$C^{14}$-ylacetamide. For example, let it be assumed that there are $a$ μg. of A in a mixture of labeled metabolites of equal molar specific activities with a total radioactivity R. Now $b$ mg. of unlabeled carrier-compound A is added to the mixture. Compound A is now reisolated and subjected to a series of rigorous and optimally specific purification procedures. After each step the specific radioactivity (s.a.) $\alpha$ of the compound is determined. When this factor is constant, the percentage of A in the mixture can be taken as $100 \times \alpha \times b/R$. In the case where $\alpha=0$, *i.e.*, where A is not radioactive after the purification procedures, it is certain that the carrier compound used was not present in the mixture analyzed. The carrier-isotope dilution technique thus yields valuable data which otherwise would not be available. It is important, however, to carry the sequence of steps sufficiently far and to be aware of the limitations of the method. Some aspects of this technique have been discussed by Heidelberger (*22*).

### *Urinary Metabolites of* N-2-*Fluorenylacetamide*

N-*2-Fluorenylacetamide.*—To a solution of 1.01 m*M* (224.5 mg.) of *N*-2-fluorenylacetamide in 7 ml. of hot ethanol was added 2 ml. of urine containing 1,520,000 c.p.m. as radioactive metabolites. On cooling, 213.7 mg. of material crystallized. This was recrystallized from a mixture of 10 ml. of ethanol and 15 ml. of water using 20 mg. of Norit. The product was then chromatographed in benzene solution on alumina (Merck, suitable for chromatographic adsorption). After removal of the solvent, the material was crystallized from 10 ml. of ethanol and 15 ml. of water to yield 132.2 mg. of *N*-2-fluorenylacetamide. Two further crystallizations from aqueous ethanol left 127.6 mg. The compound was then hydrolyzed by refluxing with 35 ml. of 3.5 *N* hydrochloric acid for 2 hours. The acid was neutralized with 140 ml. of hot 8% sodium bicarbonate solution; on cooling, 77.7 mg. of 2-fluorenamine crystallized. Reacetylation with 2 ml. of acetic anhydride in 10 ml. of benzene, followed by successive crystallizations from 20 ml. of 50% ethanol, 15 ml.

of 30% acetic acid, 15 ml. of 35% ethanol, 13, 11, and 10 ml. of 25% acetic acid, using 10 mg. of Norit on the last step, gave a product of constant s.a. of 6460 c.p.m. per m*M*. Hence, unchanged *N*-2-fluorenylacetamide accounted for 6460 × 1.01 × 100/1,520,000, or 0.43%, of the urinary radioactivity.

*2-Fluorenamine.*—To a solution of 1.23 m*M* of 2-fluorenamine in 400 ml. of boiling water was added 2 ml. of urine containing 1,520,000 c.p.m. and the hot solution was filtered. The crystalline product obtained on cooling was filtered, dried, and chromatographed in benzene solution on an alumina column. The benzene eluate was taken to dryness and the residue crystallized successively from 5 ml. of ethanol and 400 ml. of water, 50 ml. of ethanol and 200 ml. of water, and 25 ml. of ethanol and 100 ml. of water. Acetylation with 1 ml. of acetic anhydride in 20 ml. of benzene, followed by successive crystallizations from 30 ml. of 30% ethanol, 25 ml. of 30% ethanol, and 16 ml. of 25% acetic acid, gave products with a s.a. of 1120, 1000, and 1070 c.p.m. per m*M*. Thus 2-fluorenamine represented 1.23 × 1070 × 100/1,520,000, or 0.87%, of the radioactive urinary metabolites.

A parallel experiment using 1.23 m*M* of 2-fluorenamine and 2 ml. of the same urine incubated for 12 hours with bacterial β-glucuronidase [6] (820,000 units) after the addition of 0.5 ml. of 0.3 *M* acetate buffer, *p*H 6, gave a product with a s.a. of 1180 c.p.m. per m*M*.

N-(*1-Hydroxy-2-fluorenyl*)*acetamide.*—To a solution of 0.723 m*M* of *N*-(1-hydroxy-2-fluorenyl)acetamide (*28*) in 20 ml. of 0.5 *N* potassium hydroxide solution was added 500 μl. of urine containing 423,000 c.p.m. After thorough mixing, the *p*H was adjusted to 6 with 1 *N* hydrochloric acid and the resulting suspension was heated to the boiling point and cooled. The precipitate was filtered off and recrystallized twice from 12 and 6 ml., respectively, of 50% ethanol using 25 mg. of Norit. The compound was then recrystallized twice from 12 and 6 ml., respectively, of benzene, using 13 mg. of Norit on the first step. Solution in 5 ml. of benzene, treatment with 15 mg. of Norit, and addition of 2 ml. of cyclohexane to the hot solution gave, on cooling, crystals of a s.a. of 8370 c.p.m. per m*M*. Three further crystallizations from benezene-cyclohexane mixtures stabilized the s.a. at 5500, 5380, and 5260 c.p.m. per m*M*, respectively. Therefore, this compound is a metabolite of *N*-2-fluorenylacetamide, accounting for about 0.9% of the urinary activity. In several other experiments of this type, a somewhat lower proportion of the urinary activity, of the order of 0.1 to 0.2%, was ascribable to this compound.

Hydrolysis of glucuronides by incubation of the urine with glucuronidase, as described earlier, followed by carrier analysis as outlined above showed that the sum of free *N*-(1-hydroxy-2-fluorenyl)acetamide and its glucuronide represents 1.4% of the urinary radioactivity.

N-(*3-Hydroxy-2-fluorenyl*)*acetamide.*—To a hot solution of 0.963 m*M* of *N*-(3-hydroxy-2-fluorenyl)acetamide (*28*) in 22 ml. of ethanol was

[6] Purchased from Sigma Chemical Company, St. Louis, Mo.

added 1.5 ml. of urine containing 1,140,000 c.p.m. On addition of 24.5 ml. of hot water and cooling, the compound crystallized. The material was then successively recrystallized as follows: dissolved in 35 ml. of 68% ethanol, refluxed 0.5 hr. with 20 mg. of Norit, filtered, 5 ml. of water added, and ethanol boiled off to incipient crystallization; crystallized successively from 10 ml. of 50% acetic acid, 60 ml. of 33% ethanol, 120 ml. of 17% acetic acid (20 mg. Norit), 60 ml. of 33% ethanol, 60 ml. of 17% acetic acid, 45 ml. of 33% ethanol, 115 ml. of 13% acetic acid, 40 ml. of 50% ethanol (20 mg. Norit), 60 ml. of 17% acetic acid, 30 ml. of 33% ethanol, a mixture of 5 ml. of acetic acid, 5 ml. of ethanol, and 20 ml. of water, 30 ml. of 33% ethanol, 100 ml. of benzene and 15 ml. of ethanol, 32.5 ml. of 23% ethanol. The s.a. of the compound over the last 3 operations was 6100, 5300, and 5650 c.p.m. per m*M*, respectively. Hence $0.963 \times 5650 \times 100/1{,}140{,}000$, or 0.48%, of the urinary radioactivity was accounted for by this compound.

When these experiments were performed on glucuronidase-treated urines the total *N*-(3-hydroxy-2-fluorenyl)acetamide accounted for 4.5 and 7.6% on 2 different samples. Thus the glucuronic acid-conjugated compound amounted to 4 to 6%, approximately, of the urinary metabolites of *N*-2-fluorenylacetamide.

N-(*5-Hydroxy-2-fluorenyl*)*acetamide*.—To a refluxing solution of 0.772 m*M* of *N*-(5-hydroxy-2-fluorenyl)acetamide (*19*) in 220 ml. of water was added 2.0 ml. of urine (1,520,000 c.p.m.). The mixture was filtered while hot and cooled, whereupon the compound crystallized. Purification of this material was carried out as follows: successive crystallizations from 150 ml. of water (39 mg. of Norit), 120 ml. of water, 28 ml. of xylene, 22 ml. of xylene, hydrolysis to the aminohydroxy derivative by refluxing 69 mg. of the material in 10 ml. of 12 *N* hydrochloric acid for 45 minutes, recrystallization of the amine hydrochloride in 12 ml. of 6 *N* hydrochloric acid; conversion to the free amine by means of 8% sodium bicarbonate solution, crystallization from 15 ml. of 66% ethanol; solution in 11 ml. of 0.1 *N* hydrochloric acid, followed by filtration and addition of 5 ml. of 10% sodium acetate solution and acetylation by four 0.5 ml. portions of acetic anhydride while the mixture was stirred for a total of 5 hours; successive crystallizations from 22 ml. of water and from 4.5 ml. of xylene. After the last 3 steps the s.a.'s were 73,300, 64,500, and 68,200 c.p.m. per m*M*, respectively. It was concluded that this compound accounts for $0.772 \times 68{,}200 \times 100/1{,}520{,}000$, or 3.5%, of the urinary metabolites.

Glucuronidase-hydrolyzed urine investigated in a parallel experiment showed a total of 31.4% of *N*-(5-hydroxy-2-fluorenyl)acetamide. Thus 28% of this compound was present as a glucuronic acid conjugate.

N-(*7-Hydroxy-2-fluorenyl*)*acetamide*.—Urine (2.0 ml. or 1,520,000 c.p.m.) was added to a hot solution of 0.835 m*M* of *N*-(7-hydroxy-2-fluorenyl)-acetamide (*29*) in 7 ml. of 70% ethanol; on cooling the compound crystallized. It was purified by the following steps: successive recrystallizations from 10 ml. of 40% ethanol (Norit), 8 ml. of 25% acetic acid, 12 ml. of 33% ethanol, 15 ml. of 20% acetic acid (Norit), 9 ml. of 30% acetic

acid, 11 ml. of 25% ethanol, 8 ml. of 25% acetic acid; hydrolysis to the aminohydroxy derivative by refluxing in 2 ml. of ethanol and 6 ml. of 12 *N* hydrochloric acid for 1 hour; solution of the hydrochloride in 25 ml. of 0.02 *N* hydrochloric acid and treatment with Norit (freed of adsorbed air by prior boiling in water); acetylation with acetic anhydride at *p*H 4; successive recrystallizations from 10 ml. of 20% acetic acid and 6 ml. of 17% acetic acid. The s.a.'s of the compound were 90,000, 76,000, and 76,000, respectively, after the last 3 steps. Thus *N*-(7-hydroxy-2-fluorenyl) acetamide accounted for $0.835 \times 76{,}000 \times 100/1{,}520{,}000$, or 4.2%, of the urinary radioactivity.

After glucuronidase treatment the radioactivity carried by this compound increased to 13.8% of the urinary metabolites; thus 9.6% was accounted for by the glucuronic acid conjugate. In 2 other experiments 10 and 10.3%, respectively, were found for free *N*-(7-hydroxy-2-fluorenyl)-acetamide and 21% was found for the free and glucuronic acid-conjugated compound. In one case in which a dog was fed labeled *N*-2-fluoren-9-$C^{14}$-ylacetamide, 12.7 and 25%, respectively, of the urinary radioactivity were associated with free and conjugated *N*-(7-hydroxy-2-fluorenyl)-acetamide (*30*).

The excretion of free *N*-(7-hydroxy-2-fluorenyl)acetamide at various periods after a single dose of *N*-2-fluoren-9-$C^{14}$-ylacetamide is reported in table 2. It is noteworthy that this compound represents a fair amount of the urinary metabolites even when the total amount of metabolites excreted drops to low levels.

TABLE 2.—*Excretion of free* N-*(7-hydroxy-2-fluorenyl)acetamide in urine as a function of time after administration of* N-*2-fluoren-9-*$C^{14}$-*ylacetamide*

| Period* (*hours*) | Percentage of urinary metabolites | Percentage of dose administered |
|---|---|---|
| 4–10 | 9 | 0. 8 |
| 10–22 | 5. 2 | 1. 2 |
| 22–34 | 3. 9 | 1. 0 |
| 46–58 | 13. 4 | 0. 1 |
| 58–70 | 12. 0 | 0. 05 |
| 70–82 | 11. 5 | 0. 035 |
| 82–96 | 7. 3 | 0. 03 |

*Urine for the period 34 to 46 hours was not available for study.

### *Potential Metabolites Absent in Urine of Rats Fed* N-*2-Fluorenylacetamide*

In general, the procedure used with the following compounds, which were not metabolites of *N*-2-fluorenylacetamide, was the same as that employed with the substances found to be urinary metabolites. A sample of urine from rats fed the labeled carcinogen, containing 250,000 to 2,000,000 c.p.m., was added to a solution of the appropriate compound, the compound was reisolated and then purified.

N-*Methyl-2-fluorenamine.*—After a series of operations consisting of crystallization, chromatography, vacuum sublimation, and conversion to the 3,5-dinitrobenzamide, the radioactivity of the *N*-methyl-2-fluorenamine (*31, 32*) used as carrier declined to background levels, indicating that this compound was not a metabolite of *N*-2-fluorenylacetamide.

N,N-*Dimethyl-2-fluorenamine.*—Following treatments with Norit, crystallization, and chromatography, the radioactivity of this carrier (*32*) decreased to background rates.

N-*2-Fluorenyldiacetamide.*—Vacuum sublimation and repeated crystallization from aqueous ethanol and from methanol gave a product which carried no radioactivity. During purification of the material used as carrier, it was found that chromatography of benzene solutions on alumina (Merck) and neutral or acidic aluminia (Woelm) caused the hydrolysis of the *N*-2-fluorenyldiacetamide to *N*-2-fluorenylacetamide.

N-(*9-Oxo-2-fluorenyl*)*acetamide.*—Crystallization and conversion to the 2,4-dinitrophenylhydrazone were used to purify this compound which, however, did not carry radioactivity.

*2-Amino-9-fluorenone.*—Crystallization from benzene, using Norit, followed by acetylation and crystallization from aqueous ethanol gave a product free of radioactivity.

*4′-Acetamido-2-biphenylcarboxylic acid.*—Crystallization from 20% acetic acid and vacuum sublimation gave material which was inactive, indicating that it was not a metabolite of *N*-2-fluorenylacetamide.

*4′-Amino-2-biphenylcarboxylic acid.*—After crystallization from aqueous ethanol, treatment with Norit, and acetylation the activity of this carrier compound dropped to zero.

*4-Acetamido-2-biphenylcarboxylic acid.*—The carrier compound was precipitated several times from dilute ammonium hydroxide solution and crystallized, using Norit, to give material devoid of radioactivity.

## Discussion

Table 3 summarizes the results obtained in this series of experiments. Although the test used earlier for diazotizable amine (*1*) accounted for as much as 45 percent of a dose of *N-2*-fluorenylacetamide (*4*), it was recognized that alterations in the molecule probably had occurred which were not detectable by the colorimetric method. This opinion can now be substantiated, for in our experiments the sum of *N*-2-fluorenylacetamide and 2-fluorenamine represents only 1.3 percent of the urinary activity, which in turn amounts to about 65 to 75 percent of the dose administered. Similar values for *N*-2-fluorenylacetamide and 2-fluorenamine have been reported by Gutmann and Peters (*15*) after the intramuscular injection of *N*-2-fluorenylacetamide. The small proportion of the administered carcinogen which is excreted unchanged proves that most of the administered compound is metabolized. Although deacetylation of *N*-2-fluorenylacetamide occurs to a considerable extent (*5,12*), only a relatively minor amount of 2-fluorenamine is present in the urine, showing that this compound also undergoes further changes. One of these consists, of course,

in the reverse acetylation reaction which was demonstrated *in vitro* (*12*) and which also can be surmised to proceed *in vivo* by the fact that a limited portion of administered 2-fluorenamine is excreted as the amide (*14, 33, 34*). Thus, after *N*-2-fluorenylacetamide intake, the compound excreted is the sum of the unchanged material and that produced by reacetylation of 2-fluorenamine. If reacetylation is prevented by a pantothenic acid deficiency (*34*), only a very minor amount of *N*-2-fluorenylacetamide is excreted.

TABLE 3.—*Summary of compounds investigated*

| Urinary metabolites of *N*-2-fluorenylacetamide* | | Potential metabolites absent in urine of rats fed *N*-2-fluorenylacetamide |
|---|---|---|
| Compound | Percent | Compound |
| *N*-2-Fluorenylacetamide | 0. 4 | 2-Amino-9-fluorenone |
| 2-Fluorenamine | 0. 9 | *N*-(9-Oxo-2-fluorenyl)acetamide |
| *N*-(1-Hydroxy-2-fluorenyl)acetamide | 0. 1–0. 9 | *N*-Methyl-2-fluorenamine† |
| *N*-(1-Hydroxy-2-fluorenyl)acetamide glucuronide | 0. 5 | *N,N*-Dimethyl-2-fluorenamine† |
| *N*-(3-Hydroxy-2-fluorenyl)acetamide | 0. 5 | *N*-2-Fluorenyldiacetamide |
| *N*-(3-Hydroxy-2-fluorenyl)acetamide glucuronide | 4–6 | 4-Acetamido-2-biphenylcarboxylic acid† |
| *N*-(5-Hydroxy-2-fluorenyl)acetamide | 3. 5 | 4′-Acetamido-2-biphenylcarboxylic acid† |
| *N*-(5-Hydroxy-2-fluorenyl)acetamide glucuronide | 28 | 4′-Amino-2-biphenylcarboxylic acid† |
| *N*-(7-Hydroxy-2-fluorenyl)acetamide | 4–10 | |
| *N*-(7-Hydroxy-2-fluorenyl)acetamide glucuronide† | 10–12 | |
| Total | 52–63 | |

*Data expressed as percentage of total urinary radioactivity.

†This compound was synthesized for us through the courtesy of Dr. F. E. Ray, Cancer Research Laboratory, University of Florida, Gainesville.

Hydroxylation appears to occur mainly in the unsubstituted ring of *N-2*-fluorenylacetamide. About 50 percent of the total urinary radioactivity was accounted for by the free and glucuronic acid-conjugated 5- and 7-hydroxylated derivatives. Of these 2 compounds, the former has an appreciably higher solubility in water as well as in organic solvents. Therefore, *N*-(7-hydroxy-2-fluorenyl)acetamide was the only compound isolated from urine by Bielschowsky's technique (*18*) even though the 5-hydroxy derivative was present in amounts of the same order of magnitude. Both compounds respond positively in the diazo test of Westfall and Morris (*1*). The compounds 5-hydroxy- and 7-hydroxy-2-fluorenamine give 70 and 50 percent, respectively, of the color intensity at 525 mμ (*19,27*). Thus these 2 compounds present in the concentrations listed in table 3 would show a color intensity equivalent to a 33 percent 2-fluorenamine content. This is only slightly lower than the amount of diazotizable material reported in urine of rats fed *N*-2-fluorenylacetamide either in single or multiple doses (*4,7*). Hence, an appreciable proportion of diazotizable material is accounted for by these 2 compounds.

The nitrite test was developed for the estimation of *N*-(7-hydroxy-2-fluorenyl)acetamide (*27*), but the 5-hydroxy derivative interferes in this test to the extent of 35 percent. Neither 1-hydroxy- nor 3-hydroxy-2-

fluorenylacetamide responds to the test for diazotizable amine (*1*), and they cause only slight interference in the nitrite test (*27*), so that the presence of these metabolites was not evidenced by colorimetric methods. Since our data suggest that other as yet unknown metabolites of *N*-2-fluorenylacetamide exist which still contain a diazotizable aromatic amino group, it seems prudent to interpret only in a general manner the results obtained by the diazotizable amine and by the nitrite tests.

The formation of *ortho*-hydroxylated aromatic amines has been implicated in the carcinogenic process (*35,36*). The finding of this type of compound as a metabolite of *N*-2-fluorenylacetamide in the form of both possible *ortho* isomers, *N*-(1-hydroxy-2-fluorenyl)acetamide and *N*-(3-hydroxy-2-fluorenyl)acetamide, does not contradict such a hypothesis. The isolation of *N*-(2-hydroxy-3-fluorenyl)acetamide (*37*) following the ingestion of the slightly carcinogenic *N*-3-fluorenylacetamide (*38*), however, might suggest that *ortho* hydroxylation of aromatic amines may not, *per se*, be a sufficient requirement for carcinogenesis. Although only relatively small amounts of the *ortho*-hydroxy metabolites occur, they may, nevertheless, be important in the carcinogenic action of *N*-2-fluorenylacetamide.

It is of some interest that both *ortho*-hydroxyaminofluorenes are produced, whereas in the case of 2-naphthylamine only the 1-hydroxy derivative has been isolated. One explanation could be that bond fixation (*39*) in 2-naphthylamine tends to restrict the distribution of the negative charges of the nitrogen atom to carbons 1, 6, and 8 so that hydroxylation at those positions might take place preferentially. On the other hand, the evidence available (*40–42*) suggests that bond fixation does not occur in fluorene, so that an amino group substituted in the 2 position would tend to activate at least the 1, 3, 5, and 7 positions (*19*). This hypothesis is supported by the demonstration that the rat hydroxylates all of these positions. Furthermore, the presence in the urine of many hydroxy derivates of *N*-2-fluorenylacetamide, whereas only one has actually been isolated, suggests that more sensitive techniques might lead to the discovery of several hydroxylated metabolic products in the case of other complex aromatic ring systems.

It is noteworthy that although the phenols excreted in the urine as glucuronic acid conjugates were never isolated directly from glucuronidase-treated urine, free *N*-(7-hydroxy-2-fluorenyl)acetamide (*18*) has been recovered from untreated urine. According to our data the free 7-hydroxy derivative is excreted in larger amounts than the other free hydroxy metabolites, and it is the only one to crystallize because of its lower solubility. However, after enzymic hydrolysis of the glucuronides the 5-hydroxy derivative is present in a larger amount, and the other components of the mixture then amount to a more appreciable fraction and may prevent the crystallization of any of the compounds. Bielschowsky's method (*18*) permits the isolation of *N*-(7-hydroxy-2-fluorenyl)acetamide from the urine of rats and dogs (*30*) fed *N*-2-fluorenylacetamide with fair reproducibility. However, both the poor yield of this compound and

solvent-partition experiments previously reported (*6,9*) indicate that other metabolites must be present. Our attempts to isolate these compounds by procedures patterned after those of Berenbom and Young (*43*) gave rise to untractable gums and tars which failed to yield crystalline materials. This is understandable in the light of the large number of different yet closely related compounds that we found to be urinary metabolites of *N*-2-fluorenylacetamide.

Similar phenomena were experienced during work on the metabolites of *N*-3-fluorenylacetamide (*37*), suggesting that compounds other than the reported *N*-(2-hydroxy-3-fluorenyl)acetamide were present as glucuronic acid conjugates.

In an abstract Boyland (*44*) reported 2-naphthylamine-*N*-glucosiduronic acid as a metabolite of 2-naphthylamine. Our data with 2-fluorenamine might suggest the presence of a similar conjugate. On the other hand, the values of the respective specific activities, 1180 and 1070 c.p.m. per m*M* are so close as to be within the experimental error of this type of experiment, so that the question must remain open pending further investigation.

Besides conjugation with glucuronic acid, ethereal sulfate formation is another method whereby excretion of phenols takes place (*21*). Chromatographic evidence to be reported in the following paper indicates that *N*-2-fluorenylacetamide is metabolized to sulfuric acid conjugates. Quantitatively, these conjugates are a relatively unimportant route of excretion, however, since their sum amounts to only 7 to 8 percent of total urinary radioactivity.

The presence of mercapturic acids derived from *N*-2-fluorenylacetamide in the urine of rats given an oral dose of the carcinogen was investigated by the technique of Stekol (*45*). Within the limitations of the method no evidence for such metabolites was found.

The metabolites of *N*-2-fluorenylacetamide discussed so far, which amount to a substantial portion of the urinary radioactivity, are more acidic than the compound fed. Hence, it would be expected that such compounds would extract into the organic phase of an ether-water system at acidic or neutral *p*H values and would tend to remain in a basic aqueous layer. On the whole, such behavior was observed in our ether-extraction experiments (*6,9*). However, Gutmann and Peters (*15*) indicated that considerable radioactivity was extracted by ether at *p*H 11 to 12, while relatively small amounts were ether soluble at *p*H 2 to 3, and only traces were removed at neutral *p*H. The explanation for this discrepancy is not obvious.

Our experiments indicate further that *N*-methyl-2-fluorenamine, *N,N*-dimethyl-2-fluorenamine, and *N*-2-fluorenyldiacetamide were not urinary metabolites of *N*-2-fluorenylacetamide. In addition, *N,N*-dimethyl-2-fluorenamine was not present in the urine of rats given *N*-methyl-2-fluorenamine (*32*). Thus, it can be concluded that the aromatic amino group was not methylated or diacetylated (*12*), unless these derivatives were produced in the tissues but not excreted in the urine. One such case has

been recorded with *p*-phenylazoaniline, where the methyl derivatives were detected in the livers of rats fed the dye but not in the urine (*46*). However, because of the high sensitivity of the isotope-dilution procedure a level of radioactivity corresponding to 0.001 μg. of *N*-2-fluoren-9-$C^{14}$-ylacetamide in a sample could be detected with considerable accuracy, and data showing the presence of one half that amount can be accepted with some confidence. Hence, the excretion of any small fraction of a tissue metabolite should be discernible.

Likewise, oxidation at the 9-carbon to the fluorenone derivatives did not seem to take place. Therefore, it is not surprising that the corresponding biphenylcarboxylic acid derivatives were not metabolites either.

It thus appears that hydroxylation at selected positions of the *N*-2-fluorenylacetamide ring system, with subsequent conjugation of a portion of the hydroxylated derivatives with glucuronic or sulfuric acid, is a major biochemical reaction occurring during the metabolism of the carcinogen. These products account for 50 to 60 percent of the total radioactivity excreted in the urine during a 48-hour period after a single dose of *N*-2-fluoren-9-$C^{14}$-ylacetamide.

Only 2 of the metabolites reported herein have so far been tested for carcinogenicity. 2-Fluorenamine appeared to have an effect rather similar to that of *N*-2-fluorenylacetamide itself, although some minor differences were noted. 2-Fluorenamine induced a few tumors at the site of application as well as at other sites when painted on the skin of rats, whereas *N*-2-fluorenylacetamide was carcinogenic only at other sites, such as the liver, mammary gland, etc. (*47–49*). This might be interpreted to mean that 2-fluorenamine is closer to a "primary carcinogen" than *N*-2-fluorenylacetamide since metabolism of the latter may be required to evoke the effect. This view finds support in the report (*50*) that 2-*p*-toluenesulfonamidofluorene, which cannot be hydrolyzed to the amine *in vivo*, is not carcinogenic (*51*). The 2-benzoylamino derivative, which is hydrolyzed only with difficulty (*14,15*), is only slightly carcinogenic (*51*). On the other hand, the conversion of *N*-2-fluorenylacetamide to 2-fluorenamine is reversible (*12*), and it is therefore difficult to ascribe the carcinogenic effect to one compound without implicating the other until further studies on the mechanism of action of these compounds resolve this question.

In the first evaluation of the carcinogenic effect of *N*-(7-hydroxy-2-fluorenyl)acetamide, negative results were obtained by Bielschowsky (*52*) after the oral administration of this compound to albino rats for 63 weeks. Hoch-Ligeti (*53*) reported neoplastic changes in 7 of 8 rats after intake of this compound for 100 weeks (except for 1 rat showing earlier tumors, after 54 weeks). In view of the long delay in the appearance of tumors in most of these animals it is not certain that the observed lesions were not of a spontaneous nature (*38*).

The systematic study of the carcinogenicity of the metabolic products of *N*-2-fluorenylacetamide, now in progress in this laboratory, should in time establish which chemical structures are primarily responsible for the

neoplastic transformations. However, the metabolites *per se* might not be carcinogenic, but their formation could alter the normal metabolic pattern and hence lead to malignancy (*12,14*). In this case the chemical structure of the metabolites might furnish a clue regarding the enzyme systems or cellular locations involved.

## Summary

Urinary metabolites of *N*-2-fluorenylacetamide excreted by rats after oral administration of the carcinogen were determined by the carrier-isotope dilution method. In a representative 48-hour sample of urine *N*-2-fluorenylacetamide and its deacetylated product, 2-fluorenamine, accounted for only 0.4 and 0.9 percent, respectively, of the radioactivity, indicating almost complete *in vivo* transformation of the ingested compound.

The percentages of free and glucuronic acid-conjugated metabolites were as follows (the first figure represents the free material and the second, the conjugated): *N*-(1-hydroxy-2-fluorenyl)acetamide—0.1 to 0.9, 0.5; *N*-(3-hydroxy-2-fluorenyl)acetamide—0.5, 4 to 6; *N*-(5-hydroxy-2-fluorenyl)acetamide—3.5, 28; *N*-(7-hydroxy-2-fluorenyl)acetamide—4 to 10, 10 to 12. Thus, biological hydroxylation at selected and theoretically predicted positions of the polynuclear ring system and conjugation of the hydroxylated derivatives with glucuronic acid is an important reaction in the metabolism of *N*-2-fluorenylacetamide. These metabolites account for 50 to 60 percent of the total urinary radioactivity in the rat after a single dose of *N*-2-fluoren-9-$C^{14}$-ylacetamide.

By analogy with the metabolism of other aromatic compounds, further possible transformations could involve methylation of the amino group, while oxidation of the 9-methylene carbon to a 9-ketone and splitting of the latter compound could lead to a carboxybiphenyl derivative. Within the limits of the sensitive method used, however, none of the following compounds were detected in the urine: *N*-methyl-2-fluorenamine, *N,N*-dimethyl-2-fluorenamine, *N*-2-fluorenyldiacetamide, 2-amino-9-fluorenone, *N*-(9-oxo-2-fluorenyl)acetamide, 4-acetamido-2-biphenylcarboxylic acid, 4′acetamido-2-biphenylcarboxylic acid, and 4′-amino-2-biphenylcarboxylic acid.

## References

(*1*) Westfall, B. B., and Morris, H. P.: Photometric estimation of N-acetyl-2-aminofluorene. J. Nat. Cancer Inst. 8: 17–22, 1947.

(*2*) Westfall, B. B.: Estimation of 2-aminofluorene and related compounds in biologic material. J. Nat. Cancer Inst. 6: 23–29, 1945.

(*3*) Morris, H. P., and Westfall, B. B.: Distribution of N-acetyl-2-aminofluorene in the rat following a single feeding. J. Nat. Cancer Inst. 9: 149–154, 1948.

(*4*) ———: Some studies of the excretion of diazotizable material after feeding 2-acetylaminofluorene to rats. Cancer Res. 10: 506–509, 1950.

(*5*) Weisburger, J. H., Weisburger, E. K., and Morris, H. P.: The excretion of radioactivity during a four-day period following the feeding of carbon 14-labeled 2-acetylaminofluorene to rats. J. Nat. Cancer Inst. 11: 797–803, 1951.

(*6*) WEISBURGER, E. K., WEISBURGER, J. H., and MORRIS, H. P.: Studies on the metabolism of 2-acetylaminofluorene-9-$C^{14}$. Arch. Biochem. 43: 474–484, 1953.

(*7*) DYER, H. M., DAMRON, C. M., and MORRIS, H. P.: A comparison of the recovery of isotopic and of diazotizable nitrogen following gastric intubation of 2-acetylaminofluorene-$N^{15}$ to rats. J. Nat. Cancer Inst. 14: 93–104, 1953.

(*8*) WEISBURGER, J. H., WEISBURGER, E. K., SOBER, H. A., and MORRIS, H. P.: Fractionation and identification of some metabolites of 2-acetylaminofluorene-9-$C^{14}$. (Abstract.) Fed. Proc. 12: 287, 1953.

(*9*) WEISBURGER, J. H., WEISBURGER, E. K., and MORRIS, H. P.: The effect of riboflavine on the metabolism of 2-acetylaminofluorene-9-$C^{14}$ in rats. J. Nat. Cancer Inst. 15: 37–47, 1954.

(*10*) MILLER, E. C., and MILLER, J. A.: Biochemical investigations on hepatic carcinogenesis. J. Nat. Cancer Inst. 15: 1571–1590, 1955.

(*11*) CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. F.: Isotopic Carbon. New York, John Wiley & Sons, 1949, pp. 278–282.

(*12*) PETERS, J. H., and GUTMANN, H. R.: The acetylation of 2-aminofluorene and the deacetylation and concurrent reacetylation of 2-acetylaminofluorene by rat liver slices. J. Biol. Chem. 216: 713–726, 1955.

(*13*) WEISBURGER, J. H.: The enzymic deacetylation of the carcinogen 2-acetylaminofluorene and related compounds. Biochem. et biophys. acta 16: 382–390, 1955.

(*14*) DYER, H. M.: A comparison of the patterns of excretion and the carcinogenicity in the rat of 2-acetylaminofluorene and related compounds. J. Nat. Cancer Inst. 16: 11–30, 1955.

(*15*) GUTMANN, H. R., and PETERS, J. H.: Studies on the metabolism of 2-benzoylaminofluorene-9-$C^{14}$ and 2-acetylaminofluorene-9-$C^{14}$ in the rat. Cancer Res. 13: 415–421, 1953.

(*16*) MILLER, J. A., and MILLER, E. C.: The carcinogenic aminoazo dyes. *In* Advances in Cancer Research (Greenstein, J. P., and Haddow, A., eds.). New York, Academic Press, Inc., 1953, Vol. I, pp. 339–396.

(*17*) WILLIAMS, R. T., Ed.: Biological Oxidation of Aromatic Rings. Biochemical Society Symposia, No. 5, 1950, pp. 1–96.

(*18*) BIELSCHOWSKY, F.: A metabolite of 2-acetamidofluorene. Biochem. J. 39: 287–289, 1945.

(*19*) WEISBURGER, E. K., and WEISBURGER, J. H.: 2-Acetylamino-5-hydroxyfluorene, a metabolite of the carcinogen 2-acetylaminofluorene. J. Org. Chem. 20: 1396–1401, 1955.

(*20*) BRAY, H. G., and THORPE, W. V.: Analysis of phenolic compounds of interest in metabolism. *In* Methods of Biochemical Analysis (Glick, D., ed.). New York, Interscience Publishers, Inc., 1954, Vol. I, pp. 27–52.

(*21*) WILLIAMS, R. T.: Detoxication Mechanisms. New York, John Wiley & Sons, 1947, 288 pp.

(*22*) HEIDELBERGER, C., HADLER, H. I., and WOLF, G.: The metabolic degradation in the mouse of 1,2,5,6-dibenzanthracene-9,10-$C^{14}$. III. Some quinone metabolites retaining the intact ring system. J. Am. Chem. Soc. 75: 1303–1308, 1953.

(*23*) BHARGAVA, P. M., HADLER, H. I., and HEIDELBERGER, C.: Studies on the structure of the skin protein-bound compounds following topical application of 1,2,5,6-dibenzanthracene-9,10-$C^{14}$. I. 2-Phenylphenanthrene-3,2′-dicarboxylic acid, a degradation product. J. Am. Chem. Soc. 77: 2877–2886, 1955.

(*24*) MORRIS, H. P., DUBNIK, C. S., DUNN, T. B., and WESTFALL, B. B.: Some observations on carcinogenicity, distribution, and metabolism of N-acetyl-2-aminofluorene in the rat. Acta Unio internat. contra cancrum 6: 47–51, 1948.

(*25*) WEISBURGER, J. H., and LIPNER, H. J.: Which $I^{131}$ counting system is best for laboratory use? Nucleonics 12: No. 5, 21–23, 1954.

(*26*) Robinson, C. V.: Windowless, flow type, proportional counter for counting $C^{14}$. Science 112: 198–199, 1950.

(*27*) Damron, C. M., and Dyer, H. M.: A method for the quantitative spectrophotometric measurement of 7-hydroxy-2-acetylaminofluorene. J. Nat. Cancer Inst. 14: 279–289, 1953.

(*28*) Weisburger, E. K., and Weisburger, J. H.: *ortho*-Hydroxy derivatives of the carcinogen 2-acetylaminofluorene. J. Org. Chem. 19: 964–972, 1954.

(*29*) Weisburger, J. H., and Weisburger, E. K.: 2-Acetamido-7-methoxyfluorene. J. Chem. Soc. 758–759, 1954.

(*30*) Weisburger, E. K., Weisburger, J. H., and Morris, H. P.: The metabolism of 2-acetylaminofluorene in dogs. (Abstract.) Cancer Res. 12: 305, 1952.

(*31*) Little, J. N., and Ray, F. E.: Synthesis of 2-methylaminofluorene-N-methyl-$C^{14}$. J. Am. Chem. Soc. 74: 4955–4956, 1952.

(*32*) Weisburger, E. K., Weisburger, J. H., Velat, C. A., and Morris, H. P.: The fate of the methyl group in 2-methyl-$C^{14}$-aminofluorene, including studies of the carcinogenicity of 2-methyl- and 2-dimethylaminofluorene. J. Biol. Chem. 221: 845–854, 1956.

(*33*) Gutmann, H. R., Kiely, G. E., and Klein, M.: The metabolism of 2-aminofluorene in the rat. Cancer Res. 12: 350–355, 1952.

(*34*) Allison, J. B., Wannemacher, R. W., Jr., and Migliarese, J. F.: Diet and the metabolism of 2-aminofluorene. J. Nutrition 52: 415–425, 1954.

(*35*) Clayson, D. B.: A working hypothesis for the mode of carcinogenesis of aromatic amines. Brit. J. Cancer 7: 460–471, 1953.

(*36*) Bonser, G. M., Clayson, D. B., Jull, J. W., and Pyrah, L. N.: The carcinogenic properties of 2-amino-1-naphthol hydrochloride and its parent amine 2-naphthylamine. Brit. J. Cancer 6: 412–424, 1952.

(*37*) Weisburger, E. K.: 2-Hydroxy-3-acetylaminofluorene, a metabolite of 3-acetylaminofluorene in the rat. J. Am. Chem. Soc. 77: 1914–1916, 1955.

(*38*) Morris, H. P.: Hepatocarcinogenesis by 2-acetylaminofluorene and related compounds including comments on dietary and other influences. J. Nat. Cancer Inst. 15: 1535–1547, 1955.

(*39*) Pauling, L. C.: The Nature of the Chemical Bond. Ithaca, N. Y., Cornell University Press, 1948, 450 pp., p. 142.

(*40*) Lothrop, W. C.: The structure of fluorene. J. Am. Chem. Soc. 61: 2115–2119, 1939.

(*41*) Bergmann, E., and Berlin, T.: Remarks on the structure of fluorene. J. Am. Chem. Soc. 62: 316–317, 1940.

(*42*) Weisburger, J. H., Weisburger, E. K., and Ray, F. E.: The structure of fluorene. J. Am. Chem. Soc. 72: 4250–4253, 1950.

(*43*) Berenbom, M., and Young, L.: Biochemical studies of toxic agents. 3. The isolation of 1- and 2-naphthylsulphuric acid and 1- and 2-naphthylglucuronide from the urine of rats dosed with 1- and 2-naphthol. Biochem. J. 49: 165–169, 1951.

(*44*) Boyland, E., and Manson, D.: The metabolism of 2-naphthylamine. Third Internat. Congress Biochem., p. 135, abstract 15–25, 1955.

(*45*) Stekol, J. A.: The determination of *p*-bromophenylmercapturic acid in the urine of the dog. J. Biol. Chem. 113: 279–288, 1936.

(*46*) Miller, J. A., Miller, E. C., and Baumann, C. A.: On the methylation and demethylation of certain carcinogenic azo dyes in the rat. Cancer Res. 5: 162–168, 1945.

(*47*) Morris, H. P., Dubnik, C. S., and Johnson, J. M.: Studies of the carcinogenic action in the rat of 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene after ingestion and after painting. J. Nat. Cancer Inst. 10: 1201–1213, 1950.

(*48*) Kirby, A. H. M.: The action of 2-aminofluorene in 3 pure strains of mice. Brit. J. Cancer 2: 294–300, 1948.

(*49*) LACASSAGNE, A., BUU-HOÏ, ROYER, R., RUDALI, G., and ZAJDELA, F.: Contribution à l'étude du pouvoir cancérigène du 2-aminofluorène. Compt. rend. Soc. biol. 142: 481–483, 1948.

(*50*) RAY, F. E., and ARGUS, M. F.: Studies on the metabolism, distribution, and excretion of 2-*p*-toluenesulfonamidofluorene-$S^{35}$ in the rat. Cancer Res. 11: 783–787, 1951.

(*51*) MORRIS, H. P.: Unpublished data.

(*52*) BIELSCHOWSKY, F.: The carcinogenic action of 2-acetylaminofluorene and related compounds. Brit. M. Bull. 4: 382–384, 1947.

(*53*) HOCH-LIGETI, C.: Effect of feeding 7OH-2-acetaminofluorene to albino rats. Brit. J. Cancer 1: 391–396, 1947.

# Chromatographic Separation of Some Metabolites of the Carcinogen *N*-2-Fluorenylacetamide [1]

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Ten urinary metabolites of the carcinogen *N*-2-fluorenylacetamide have been identified by means of the carrier-isotope dilution procedure (*1*). These compounds do not account for all the radioactivity in a sample of urine; hence an independent method of ascertaining the nature of these products and gaining information on as yet unknown metabolites was of considerable importance. This report details the development of chromatographic systems which permit the separation and identification of some of the metabolites of *N*-2-fluorenylacetamide.

Ether extraction of urines of rats given radioactive *N*-2-fluoren-9-$C^{14}$-ylacetamide removed only 20 to 30 percent of the radioactivity (*2,3*), whereas the compound fed was preferentially ether-soluble. This behavior indicated that a considerable proportion of the carcinogen was metabolized into more polar products. It was hoped, therefore, that the chromatographic techniques so successful in the separation of amino acids could be adapted to the fractionation of the metabolites of this carcinogen.

A variety of solvents was tried in preliminary experiments, using ascending monodimensional paper chromatograms of urines and ether extracts of urines of rats given the labeled carcinogen. Two solvent systems, 1 and 2 (table 1), gave the best separation of the water-soluble urinary metabolites of *N*-2-fluorenylacetamide. However, in these solvents chromatography of the ether-extractable metabolites gave only 2 spots of $R_f$, 0.81–0.88 and 0.91–0.95, which sometimes merged into 1 spot. This observation indicated either that only 1 or 2 metabolites were extractable into ether, or that solvent systems 1 and 2 (table 1) could not resolve a larger number of compounds. It is evident from the $R_f$ values listed in table 1 that the presently known ether-soluble metabolites of *N*-2-fluorenylacetamide could not be separated by solvent systems 1 or 2. It was concluded that the low solubility of these compounds in the aqueous

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TABLE 1.—*Mobility in 4 solvent systems and color tests applicable to some of the hydroxylated metabolites of N-2-fluorenylacetamide*

| Compound | $R_f$ values* × 100 in solvent system‡ | | | | Minimum detectable amount† and color of spot | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | F.-C.§ | Moist chlorine | | Coupling reaction | |
| | 1 | 2 | 3 | 4 | Amt. | Amt. | Color | Amt. | Color |
| *N-2*-Fluorenylacetamide | 90–98 | 90–97 | 57–72 | 48–64 | ‖ | ‖ | — | ‖ | — |
| *N*-(1-Hydroxy-2-fluorenyl)acetamide | 78–86 | 92–96 | 50–64 | 50–64 | 0. 5 | 5–10 | Orange-yellow | 0. 5 | Dull red |
| *N*-(3-Hydroxy-2-fluorenyl)acetamide | 79–88 | 91–95 | 30–45 | 39–52 | 0. 5 | 2. 5 | Pale orange | 5 | Lt. yellow |
| *N*-(5-Hydroxy-2-fluorenyl)acetamide | 88–93 | 87–92 | 12–20 | 13–25 | 0. 1 | 5 | Lt. tan | 0. 5 | Red |
| *N*-(7-Hydroxy-2-fluorenyl)acetamide | 81–87 | 84–89 | 4–10 | 5–12 | 0. 5 | 1 | Tan | 5 | Brownish |
| *N*-(8-Hydroxy-2-fluorenyl)acetamide | 84–90 | 87–91 | 6–14 | 8–17 | 0. 5 | 2. 5 | Yellow | 0. 5 | Orange-red |
| 2-Fluorenamine | 90–97 | 89–91 | 38–46 | 75–88 | 0. 1 | 2. 5 | Tan-gray | 1 | Yellow |
| 2-Amino-1-fluorenol | ¶ | 84–88 | 5–13 | 44–59 | 0. 5 | 5 | Pinkish-tan | 1 | Purplish-gray |
| 2-Amino-3-fluorenol | 93–98¶ | 84–88 | 5–13 | 44–59 | 0. 5 | 2. 5 | Pink | 5 | Tan-gray |
| 7-Amino-4-fluorenol | 84–93 | 84–88 | 1–7 | 25–41 | 0. 5 | 1 | Greenish-gray | 1 | Brown |
| 7-Amino-2-fluorenol | 78–85 | 30–85 | 1–4 | 11–22 | 0. 05 | >1 | Yellow | — | — |
| 7-Amino-1-fluorenol | 81–88 | 85–88 | 2–6 | 21–34 | 0. 5 | 1–2 | Tan | 0. 5 | Rusty red |

*Range of $R_f$ values for 20 μg. of material. The range was smaller, and thus the separation better, with lower amounts of substances.

†Minimum detectable amount, expressed in micrograms, over an area of approximately 50 mm.² All compounds gave a blue color with the Folin-Ciocalteu reagent. The coupling reaction was performed by spraying the paper with a dilute solution of diazotized 7-nitro-2-fluorenamine followed by a spray of 20 percent sodium carbonate solution.

‡Composition of solvents:

Solvent 1.—*sec.*-Butanol, 3 percent ammonium hydroxide, 3:1 parts (v:v);

Solvent 2.—*tert.*-Butanol, formic acid, water, 70:15:15 parts (v:v);

Solvent 3.—Top layer of cyclohexane, *tert.*-butanol, glacial acetic acid, water, 16:4:2:1 parts (v:v);

Solvent 4.—Top layer of cyclohexane, *tert.*-butanol, pyridine, water, 16:2:2:1 parts (v:v);

Lower layers of solvents 3 and 4 were used to equilibrate tank atmosphere for 15 hours at 23°.

§Folin-Ciocalteu reagent.

‖ The $R_f$ values were determined by the use of radioactive *N*-2-fluoren-9-$C^{14}$-ylacetamide by making autoradiographs of the chromatograms.

¶This compound decomposed completely in this solvent containing ammonium hydroxide (4). Similarly, only a trace of the 2-amino-3-fluorenol was visible after spraying with the Folin-Ciocalteu reagent.

phase was responsible for their movement near the solvent front, indicative of a partition ratio in favor of the organic phase. A systematic survey was therefore made wherein the polarity of the organic phase was decreased by increasing the proportions of solvents of successively lower polarity, such as in the series methanol, ethanol, *tert.*-butanol, *sec.*-butanol, *n*-butanol, toluene, benzene, cyclohexane, and ligroin. On the other hand, by the proper addition of small amounts of water-miscible organic solvents (acetic acid, methanol, *tert.*-butanol, and pyridine) the solubility of the compounds in the stationary phase was increased. Two of the large number of solvent systems tested (3 and 4, table 1) were found suitable for the resolution of some of the ether-soluble metabolites of *N*-2-fluorenylacetamide on paper chromatograms. Solvent system 3 was adapted to a silicic acid partition column, where separations could be effected on a larger scale.

## Materials and Methods

### *Compounds*

*N*-2-Fluorenylacetamide was used as the compound labeled in the 9 carbon atom with carbon 14 (*5,6*), since no suitable color test to reveal its position on the chromatogram could be developed. The other compounds used in the evaluation of the chromatographic methods were not labeled, since the color tests described below were sensitive enough to demonstrate the location of the compounds on the paper without overloading the chromatogram. The ether extract of urine of a rat fed *N*-2-fluorenylacetamide was expected to contain, among others, the following compounds: 2-fluorenamine, *N*-(1-hydroxy-2-fluorenyl)acetamide, *N*-(3-hydroxy-2-fluorenyl)acetamide, *N*-(5-hydroxy-2-fluorenyl)acetamide, and *N*-(7-hydroxy-2-fluorenyl)acetamide [references to the preparation of these compounds are cited in (*1*)]. The aforementioned substances served, therefore, as initial standards for the development of the chromatographic technique.

### *Color Tests*

The three procedures listed in table 1 were found useful in revealing the position of some of the metabolites of *N*-2-fluorenylacetamide.

*Folin-Ciocalteu reagent.*—This reagent, prepared exactly as described (*7*), was diluted with 3 parts of distilled water prior to use. The paper chromatograms were sprayed lightly, but uniformly, with the diluted reagent by means of an all-glass atomizer connected to a compressed-air line. The location of the compounds was revealed by a stable blue spot which developed in a few minutes at room temperature after a second spray with 20 percent sodium carbonate solution applied immediately following the first spray. This was the most sensitive test but did not discriminate between the compounds, since it depends on the production of molybdenum blue by reducing substances.

*Chlorine.*—Colors appeared immediately on the dried paper chromatograms of the test compounds upon exposure to chlorine. A contact time of 15 seconds was usually allowed. The spots were traced and recorded within an hour since the colors tended to fade to a light tan overnight. This test was most suitable for 7-amino-2-fluorenol, as it gave a characteristic yellow color with great sensitivity.

*Coupling reaction.*—This test was patterned after the coupling reactions using diazotized *p*-nitroaniline or sulfanilic acid (*8*), but more intense and stable colors were obtained with another reagent prepared as follows: Reflux 50 mg. of 7-nitro-2-fluorenamine (*9*) in 20 ml. of 0.5 *N* hydrochloric acid for 10 minutes, cool in an ice bath, add 20 mg. of sodium nitrite, and stir 15 minutes. Destroy the excess nitrite by urea over a period of 30 minutes, add a pinch of Celite, filter through a fluted filter into an atomizer, and add water to make about 100 ml. The reagent was stable for a few days when kept refrigerated. The chromatograms were sprayed with this reagent first, then with the 20 percent sodium carbonate solution, which brought out the colors on a white background. The background turned light tan overnight, but the colors were fairly stable. Those hydroxy derivatives which had a free *para* position in the same ring gave a red color with greater sensitivity than those compounds where coupling must have occurred in an *ortho* position. 7-Amino-2-fluorenol gave a negative test.

### *Metabolites of* N-2-*Fluorenylacetamide in Urine and Urinary Extracts*

Urines of rats fed the labeled carcinogen were collected in ice-cold graduated cylinders (*1*). At the end of the desired collection period the samples were boiled for 30 seconds to destroy any enzymes, such as β-glucuronidase, which might be present. Omission of this step led to progressive alteration of the urines, even when stored at 4° C., with production of more of the ether-soluble and reduction of the water-soluble metabolites.

After buffering the samples with 0.5 to 2.0 ml. of 0.3 *M* acetate buffer, *p*H 6, they were extracted with freshly distilled, peroxide-free ether in a continuous liquid-liquid extractor for 6 hours. The ether solutions were allowed to evaporate to dryness at room temperature, or alternatively were blown down in a stream of nitrogen. The residue was taken up in a small volume of ethanol for the paper chromatograms or *tert.*-butanol for the silicic acid partition columns.

Glucuronides were hydrolyzed by the addition of 5 to 50 mg. of glucuronidase [4] to the urines buffered to *p*H 6, followed by overnight incubation at 37°. Sulfuric acid conjugates were split similarly with Taka-diastase. [5]

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[4] Bacterial β-glucuronidase with a specific activity of 25,000 units per milligram was purchased from Sigma Chemical Company, St. Louis, Mo.

[5] We are grateful to Professor R. T. Williams, St. Mary's Hospital Medical School, London, for suggesting this method. Taka-diastase was purchased from Parke-Davis & Company, Detroit, Mich.

### *Chromatography*

*Paper.*—In general Schleicher and Schuell grade No. 598 paper was used throughout this work; although grade No. 596 gave sharper bands, it had the disadvantage of overloading readily. For 1-dimensional chromatograms, grids of eighteen 7-mm.-wide strips were cut in a 14 × 17 inch sheet of paper with an automatic cutter (*10*). The strips prevented the sidewise movement of the applied materials and therefore permitted the use of smaller amounts without danger of loss over a large area. Furthermore, more substances could be run simultaneously on the same sheet of paper than would be feasible on a plain sheet.

*Procedure and solvents.*—Materials to be chromatographed were applied 1 to each strip 2½ inches from the bottom of the sheet. This distance was preferable to a shorter one because of the fast rise of the solvent, and consequent lack of equilibrium conditions, in the initial stages of the chromatogram. A nick with a diamond stylus rather than a pencil line was used to trace the starting line because some of the metabolites of *N*-2-fluorenylacetamide were irreversibly adsorbed on the carbon-containing pencil tracing.

Table 1 lists the composition of the mixtures which were prepared immediately prior to use.

Solvents 1 and 2 consisted of a single phase. The chromatograms with these systems were run in an ascending-descending fashion for 18 to 24 hours in an aquarium type of tank as described by Block *et al.* (*11*). Solvents 3 and 4 were made up in a separatory funnel and equilibrated at the temperature of the chromatographic development. The aqueous phase was placed in a small beaker or petri dish, containing a piece of tissue paper, in the center of a museum jar (7⅝ inches in diameter and 18 inches high). A few ml. of the organic phase was placed at the bottom of the jar and the paper was suspended above the liquid level for overnight equilibration. Omission of this step led to nonreproducible $R_f$ values. The solvent was then introduced to the bottom of the museum jar by means of a long tube so that about ½ inch of the lower end of the paper dipped into the solvent. Six to 8 hours was generally required to move the solvent front in an ascending fashion about 12 inches beyond the point of application of the substances to be separated. The papers were then dried in a few minutes by the air stream of a hood: however, overnight aeration was required for the complete removal of traces of acetic acid or pyridine. The solvent front was visible on the dry papers in an ultraviolet light [6] but none of the metabolites could be visualized thus.

*Technique for radioactive samples.*—The radioactivity of liquid samples, such as urines, extracts, and paper or column eluates, was determined by plating of aliquots on 1.5-inch diameter glass disks (*12*) followed by counting in a windowless counter having 48 percent efficiency. Except for 2-fluorenamine, the metabolites of *N*-2-fluorenylacetamide were not

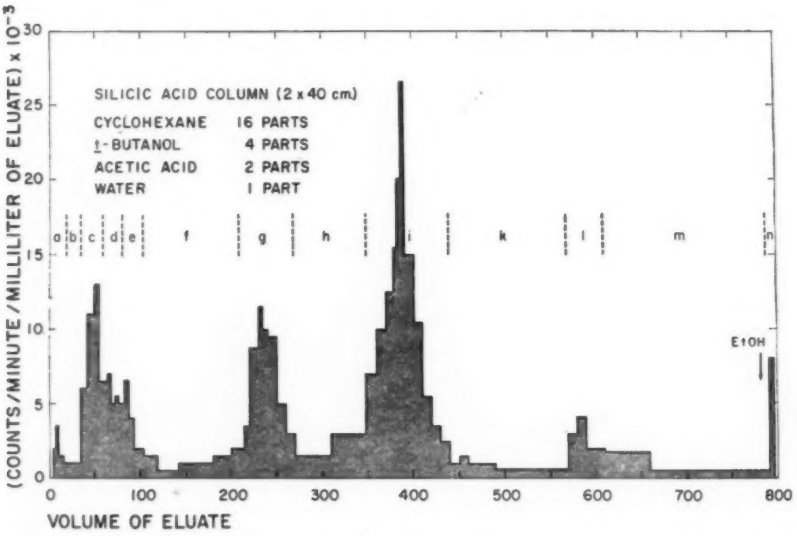
[6] Hanovia Mineralight #30600, Hanovia Chemical and Manufacturing Company, Newark, N. J.

volatile under these conditions, and no loss of radioactivity was encountered. Conversion of 2-fluorenamine to the hydrochloride by addition of a trace of hydrochloric acid also rendered this compound nonvolatile.

Application of 5 to 75 μl. samples containing 5,000 to 20,000 counts per minute (c.p.m.), as determined by the above-mentioned procedure, to paper strips resulted in spots having a count rate of 200 to 600 c.p.m. when placed directly under the window of an end-window counter.[7]

Autoradiographs of the developed chromatograms were prepared by placing the paper in firm contact with a 14×17-inch sheet of Kodak No-Screen X-ray film in a commercial X-ray cassette. Exposures of 1 to 2 weeks, depending on the number and sharpness of the spots, were usually required with the amount of radioactivity indicated above to obtain satisfactory autoradiographs. Chromatograms containing both dense and weak spots were exposed between 2 sheets of film. The first sheet was carefully removed after 1 week and the second film was developed after 4 or more weeks, depending on the results shown by the first sheet. Film and cassettes were stored in a cold room to keep the background low. The exposed film was processed in the usual manner.

Examination of the chromatograms by autoradiography was preferred over the scanning with a counter because better resolution of closely spaced spots with very different relative activities, such as encountered in this work, was achieved by the film technique.




TEXT-FIGURE 1.—Effluent diagram of ether-soluble urinary metabolites of *N*-2-fluoren-9-$C^{14}$-ylacetamide. Experimental conditions are detailed in text.

[7] Nuclear-Chicago model D 34, window thickness 1.4 mg. per cm.² or equivalent counter.

*Chromatographic separation on a column.*—A typical chromatogram using solvent system 3 will be described. Sixty grams of silicic acid (Mallinckrodt, 100 mesh) was intimately mixed with 30 ml. of the aqueous phase of the solvent mixture and transferred to a 2×40 cm. chromatographic tube (Corning #38450) as a slurry in the filtered organic layer. After settling under a pressure of 5 lbs. of nitrogen, the column, which was 30 cm. high, was allowed to stand overnight while covered with solvent. The butanol solution (2.2 million c.p.m. in 0.85 ml.) of an ether extract of glucuronidase-treated urine containing radioactive metabolites of *N*-2-fluoren-9-$C^{14}$-ylacetamide was carefully applied to to the top of the column and washed in with two 1 ml. portions of the solvent. A solvent reservoir containing the organic phase was then attached to the top of the chromatographic tube through a ball joint and a nitrogen pressure of 2 to 3 lbs. was applied. Two ml. fractions of eluate were collected every half hour by means of an automatic fraction collector. Fifty μl. aliquots of each tube were plated and counted. When no further radioactivity appeared the column was washed with ethanol, which gave rise to another peak. A plot of radioactivity against volume of eluate is shown in text-figure 1. Suitable fractions under peaks were combined as indicated, and aliquots were chromatographed on paper together with known compounds to identify the components.

## Results

*Paper chromatography.*—It is apparent that solvent systems 1 and 2 are not suitable for the separation of any of the compounds listed in table 1. Solvents 3 and 4, on the other hand, yield a fair resolution of these compounds, provided that the hydroxyfluorenamines can be separated from the acetyl derivatives by fractionation prior to chromatography. This can be achieved readily by extraction of an ether solution of a mixture of these compounds with very dilute hydrochloric acid, which removes the free amines. The acetylated compounds have approximately the same $R_f$ values in either of the solvents, while the free amines move at different rates in solvents 3 and 4.

The rate of travel of the compounds in these solvents was quite reproducible provided that care was taken to establish and maintain equilibrium conditions. Equilibration for 4 hours instead of overnight or variation in temperatures resulted in different $R_f$ values consisting, usually, of a somewhat faster movement of the hydroxy derivatives. However, separation of the compounds from each other was still achieved under those conditions. Thus, $R_f$ values of 0.75–0.85, 0.70–0.82, 0.45–0.51, 0.23–0.33, 0.10–0.21, and 0.05–0.16 were occasionally encountered for *N*-2-fluorenylacetamide and the 1-, 3-, 5-, 8-, and 7-hydroxy derivatives, respectively, in solvent system 3.[8] When chromatograms of unknown mixtures were run, the known compounds were routinely chromatographed simultaneously so as to detect any irregularity.

[8] The higher mobilities were found when the solvent traveled in the machine direction of the paper, while the lower $R_f$ values were obtained when the paper was cut at right angles to this direction.

A typical autoradiograph of a chromatogram of an ether extract of urines containing the radioactive metabolites of the labeled carcinogen showed intense spots with $R_f$ values of 0.01–0.06, 0.08–0.11, 0.13–0.20, 0.30–0.50, 0.55–0.75, as well as some areas with small amounts of radioactivity. The spot with an $R_f$ of 0.75 was rather elongated and probably represented both *N*-2-fluorenylacetamide and the 1-hydroxy derivative. The mobility of the other unknown spots agreed with that of the corresponding known compounds listed in table 1.

*N*-(8-Hydroxy-2-fluorenyl)acetamide was recently shown to be a minor metabolite of *N*-2-fluorenylacetamide (*13,14*). This compound, however, could not be demonstrated directly on the chromatograms of urine containing the metabolites of the carcinogen because its $R_f$ value places it between the 5- and 7-hydroxy derivatives, which are present in relatively much larger amounts. Thus the 8-hydroxy derivative generally is masked. Nevertheless, direct evidence that this compound is a metabolite of the carcinogen can be adduced from the column-chromatographic technique, which affords a prior separation, followed by paper chromatography (*see* below).

In general, solvent system 4 was less suitable than 3 for the resolution of the hydroxylated metabolites of *N*-2-fluorenylacetamide. The band representing the 1-hydroxy derivative overlapped that of the 3-hydroxy compound so that resolution was impaired unless very small amounts of the compounds were present in a mixture. Both solvents were used in 2-dimensional chromatograms, giving a better separation of the individual components described above than in a 1-dimensional run. However, no other spots appeared, indicating no other components were present within the limitations of the resolving power of the individual solvent systems.

*Column chromatography.*—Table 2 lists those peaks of the effluent from the column which could be identified by the simultaneous paper chromatography of known compounds and unknown fractions. The solution in peak *c* corresponded to *N*-(1-hydroxy-2-fluorenyl)acetamide. However, *N*-2-fluorenylacetamide, which is present according to the carrier experiments (*1*), may also be contained in this fraction since the solvent system discriminates between these 2 compounds with difficulty. Peak *e* consisted of the 3-hydroxy derivative, while the 5-hydroxy derivative was cleanly separated under peak *g*. The 8-hydroxy derivative was contained in peak *h*, but was not separated very sharply from the succeeding peak *i*, which contained a relatively large amount of the 7-hydroxy compound. A carrier-isotope analysis on the fraction under peak *h* by suitable methods, such as detailed in the previous paper (*1*), showed that this fraction contained 60 percent of the 8-hydroxy derivative. The components constituting the other peaks visible in text-figure 1 are at present not definitely known, and further work is under way to identify them. Thus, the column method not only separated the hydroxylated derivatives of *N*-2-fluorenylacetamide which have so far been identified, but also afforded a method by which larger amounts

of unknown metabolites can be made available for study. Some clues regarding the structure of the unknowns can be derived from their position on the column, since, under the experimental conditions used, the substances are eluted in the order of increasing polarity.

TABLE 2.—*Identity of fractions separated on silicic acid column*

| Peak | Effluent volume fraction (*ml.*) | Compound |
|---|---|---|
| *c* | 36–60 | *N*-(1-Hydroxy-2-fluorenyl)acetamide |
| *e* | 82–104 | *N*-(3-Hydroxy-2-fluorenyl)acetamide |
| *g* | 210–270 | *N*-(5-Hydroxy-2-fluorenyl)acetamide |
| *h* | 270–350 | *N*-(8-Hydroxy-2-fluorenyl)acetamide |
| *i* | 350–440 | *N*-(7-Hydroxy-2-fluorenyl)acetamide |

*An application of the chromatographic method—sulfuric acid conjugates as metabolites of* N-*2-fluorenylacetamide.*—Ether extraction of urine containing the metabolites of the labeled carcinogen removed 30 percent of the radioactivity as the free hydroxylated derivatives and left 70 percent in the aqueous phase. Aliquots of the latter were incubated with β-glucuronidase, Taka-diastase, and β-glucuronidase plus Taka-diastase. The resulting solutions were extracted again with ether to remove the materials freed by the enzymic hydrolysis. Ten percent of the activity was hydrolyzed by Taka-diastase (table 3) and may have been associated with sulfuric acid conjugates, which thus would amount to 7 percent of the total urinary metabolites. The ether extracts were processed as described in the experimental part and aliquots were chromatographed on paper in solvents 3 and 4. The autoradiographs from paper chromatograms of the ether extracts of urine and glucuronidase-treated urine were similar to those obtained in the previously described experiments. The chromatograms of the Taka-diastase-hydrolyzed urine were noteworthy by the absence of the normally intense spot corresponding to *N*-(5-hydroxy-2-fluorenyl)acetamide. There were only questionable traces of the compounds having $R_f$ values corresponding to the 1- and 3-hydroxy derivatives. *N*-(7-Hydroxy-2-fluorenyl)acetamide was the main radioactive spot, although some radioactivity also remained at or near the

TABLE 3.—*Ether-extractable urinary metabolites of* N-*2-fluorenylacetamide after hydrolysis by various enzymes**

| Enzyme preparation | Percentage ether-extractable |
|---|---|
| β-Glucuronidase | 53 |
| Taka-diastase | 10 |
| β-Glucuronidase and Taka-diastase | 63 |

*The aqueous phase (460,000 c.p.m.) of an ether-extracted urine was incubated at 37° with 35 mg. of each enzyme preparation for 9 hours.

origin. Thus, conjugation with sulfuric acid seemed to be more selective than that with glucuronic acid, which occurred to a considerable extent with all the hydroxylated derivatives so far identified.

The experiments with the Taka-diastase-hydrolyzed urine can be interpreted, however, in 2 ways. The complete absence of *N*-(5-hydroxy-2-fluorenyl)acetamide in the hydrolysate might indicate that the rat does not conjugate this compound with sulfuric acid or that the conjugate is not hydrolyzed by the enzyme preparation used in our experiments. If the observation of Boyland *et al.* (*15*) that *ortho*-amino hydroxysulfates could not be split by Taka-diastase were applicable to the fluorene derivatives, the 5-hydroxy conjugate should be hydrolyzed and the 1- and 3-hydroxy derivatives should not.

## Discussion

The chromatographic procedures thus confirm by a direct approach the presence of all 5 hydroxylated derivatives of *N*-2-fluorenylacetamide which have been shown to occur as metabolites of the carcinogen by the carrier-isotope dilution procedure. Therefore, the concordant results obtained by 2 different methods constitute suitable evidence for the hydroxylation at positions 1, 3, 5, 7, and 8 of the fluorene ring system. A comparison of the quantitative data obtained by the 2 approaches, however, reveals certain discrepancies which remain to be resolved. The carrier-isotope procedure consistently showed that *N*-(1-hydroxy-2-fluorenyl)acetamide was present in smaller amounts than the 3-hydroxy derivative. Yet, the spot on the paper chromatograms as well as the column fractionations indicated with equal reproducibility that the area corresponding to the 1-hydroxy derivative was more intense than that due to the 3-isomer. This suggests that there are other metabolites having mobilities similar to *N*-(1-hydroxy-2-fluorenyl)acetamide under the conditions of our experiments. Carrier experiments (*16*) demonstrated that 7-amino-2-fluorenol is a very minor metabolite of *N*-2-fluorenylacetamide. However, the spot on paper chromatograms in solvent 3 with an $R_f$ value of 0.06 (corresponding to 7-amino-2-fluorenol) represented a larger amount of radioactivity than that expected on the basis of the carrier experiments. Hence, it must be assumed that there is at least 1 as yet unknown metabolite with an $R_f$ value of 0.06.

Similarly, the weak bands occurring in other regions of the chromatograms might indicate the presence of other minor metabolites, while the slow-moving components near the origin on the paper chromatograms and the last eluted fraction from the column represent appreciable unknown portions of the ether-extractable urinary metabolites of *N*-2-fluorenylacetamide. In view of their position on the chromatograms, these compounds must be quite polar.

The paper and column techniques yield separation of the ether-extractable metabolites of the carcinogen. The paper-chromatographic procedure is considerably faster than the column for routine application, such as determination of the composition of extracts in terms of the main

metabolites. On the other hand, substantial amounts of the metabolites can be separated on the column. Hence, those minor constituents of the mixture which might be overlooked on the paper chromatogram are separated somewhat better and account for a sufficiently large number of counts so that their presence is revealed by the column method. For example, the recently discovered metabolite of *N*-2-fluorenylacetamide, *N*-(8-hydroxy-2-fluorenyl)acetamide (*13*), was masked on paper chromatograms by the neighboring stronger spots due to the 5- and 7-hydroxy derivatives. Chromatography on the column, however, had better resolving power, so that the 8-hydroxy derivative appeared as a discrete entity. Moreover, only the column yields sufficient material to undertake the identification of unknown metabolites.

Similar techniques are being applied to the separation of the metabolites of *N*-2-fluorenylacetamide obtained from tissues and also to the fractionation of the water-soluble metabolites.

## Summary

Two new solvent systems have been developed for the separation on paper chromatograms of some of the ether-soluble urinary metabolites of the carcinogen *N*-2-fluorenylacetamide. One of these mixtures has been adapted to a partition-column procedure on silicic acid permitting the resolution of these compounds on a larger scale. Thus, the isomeric *N*-(1-, 3-, 5-, 7-, and 8-hydroxy-2-fluorenyl)acetamides were separated and identified chromatographically.

The chromatographic procedure was also applied to a study of the sulfuric acid conjugates of the above-mentioned hydroxylated derivatives. The evidence obtained indicated that a rat fed *N*-2-fluorenylacetamide excreted in the urine mostly the sulfate of the 7-hydroxy derivative but none of the 5-hydroxy derivative. This conclusion, however, is based on the assumption that Taka-diastase could hydrolyze the sulfuric acid conjugate of the 5-hydroxy derivative if it were present.

## References

(*1*) Weisburger, J. H., Weisburger, E. K., and Morris, H. P.: Urinary metabolites of the carcinogen *N*-2-fluorenylacetamide. J. Nat. Cancer Inst. 17: 345–361, 1956.

(*2*) Weisburger, E. K., Weisburger, J. H., and Morris, H. P.: Studies on the metabolism of 2-acetylaminofluorene-9-$C^{14}$. Arch. Biochem. 43: 474–484, 1953.

(*3*) Weisburger, J. H., Weisburger, E. K., and Morris, H. P.: The effect of riboflavine on the metabolism of 2-acetylaminofluorene-9-$C^{14}$ in rats. J. Nat. Cancer Inst. 15: 37–47, 1954.

(*4*) Weisburger, E. K., and Weisburger, J. H.: *ortho*-Hydroxy derivatives of the carcinogen 2-acetylaminofluorene. J. Org. Chem. 19: 964–972, 1954.

(*5*) Ray, F. E., and Geiser, R. C.: Synthesis of 2-acetylaminofluorene-9-$C^{14}$ and 2-acetylaminofluorene-ω-$C^{14}$. Cancer Res. 10: 616–619, 1950.

(*6*) Heidelberger, C., and Rieke, H. S.: The synthesis of 3,4-benzpyrene-5-$C^{14}$ and of 2-acetylaminofluorene-9-$C^{14}$. Cancer Res. 11: 640–643, 1951.

(7) SNELL, F. D., and SNELL, C. T.: Colorimetric Methods of Analysis. New York, D. Van Nostrand Co., Inc., Vol. III, Part I, 1953, p. 116.

(8) *Ibid.*, p. 117.

(9) WEISBURGER, J. H., and WEISBURGER, E. K.: 2-Acetamido-7-methoxyfluorene. J. Chem. Soc. 758–759, 1954.

(10) SOBER, H. A.: Unpublished design.

(11) BLOCK, R. J., DURRUM, E. L., and ZWEIG, G.: A Manual of Paper Chromatography and Paper Electrophoresis. New York, Academic Press, Inc., 1955, p. 25.

(12) CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. F.: Isotopic Carbon. New York, John Wiley & Sons, Inc., 1949, p. 113.

(13) WEISBURGER, K. E., and WEISBURGER, J. H.: Synthesis of *N*-(8-hydroxy-2-fluorenyl)acetamide. J. Org. Chem. In press.

(14) WEISBURGER, E. K., WEISBURGER, J. H., and MORRIS, H. P.: *N*-(8-Hydroxy-2-fluorenyl)acetamide, a minor metabolite of *N*-2-fluorenylacetamide in the rat. Presented at meeting of the American Chemical Society, Atlantic City, Fall 1956, Abstract of Papers, p. 2C.

(15) BOYLAND, E., MANSON, D., SIMS, P., and WILLIAMS, D. C.: The biochemistry of aromatic amines. The resistance of some *o*-aminoaryl sulphates to hydrolysis by aryl sulphatases. Biochem. J. 62: 68–71, 1956.

(16) WEISBURGER, E. K., WEISBURGER, J. H., and MORRIS, H. P.: Unpublished data.

# The Components of Cigarette Smoke. I. Preliminary Studies [1, 2]

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Wynder, Graham, and Croninger (*1*) have demonstrated that the application of cigarette-smoke condensates to the skin of mice and rabbits elicits carcinomas at the site of application. This paper reports the primary chemical phase of an attempt to isolate and identify the substance or substances in tobacco smoke responsible for the observed biological activity. The presence of numerous compounds has been reported in tobacco smoke (*2*), but a large part of such condensates consists of unidentified material.

## Experimental

### *Smoking Machine*

The apparatus (plate 25) accommodates 240 cigarettes in 12 manifolds; the cigarettes are positioned horizontally and the distance between their axes is 25 mm. The manifolds are connected to 2 Y-tubes (6 per Y) by means of ball-and-socket joints, and these Y's lead in turn into a larger Y-tube which is fixed to the collecting system. This last consists of 4 cold traps in series, the first being a cylindrical vessel with an inner tube of large (5 cm.) diameter, the second and third, balloon-type flasks of about 1 liter capacity, and the fourth, a double U-tube. All glass connections in the apparatus are through standard taper or ball-and-socket ground joints. The first, third, and fourth traps are immersed in liquid air and the second in dry ice-acetone. The vacuum is generated by a Crowell rotary air pump, size 2-A. A 25-gallon reservoir tank is in parallel with the pump, and needle valves in the connecting line to the system control the flow of air. Two solenoid valves opening simultaneously, in parallel, are actuated by a Haydon timing motor and connect the pump system to the fourth collecting trap. (The function of a second solenoid valve is merely to increase the area of the orifice between the vacuum system and the smoking section.)

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[2] Aided by grants from the American Cancer Society and Damon Runyon Memorial Fund.
[3] We wish to thank Professor Norton Nelson for his advice and encouragement during the course of this investigation. We are indebted to Professor O. R. Trautz for the X-ray diffraction data and their interpretation.

The machine is adjusted to take a 2-second puff once a minute (*3*). The average volume of each puff is in the range of 35 to 40 ml. per cigarette, depending upon the length of the cigarette and the amount of tar deposited in the collection train. Inasmuch as variations in the physical structure of individual cigarettes exist, the air drawn through each cigarette will not be uniform. From kymograph measurements made with a Gorrell and Gorrell Mono-Drum and tambour inserted in the system, a pressure-time curve for the puffing process was determined; the pressure value reaches a maximum (*ca.* 40 mm. $H_2O$) almost instantly, falls off slowly, and then very quickly drops off to zero at the end of the puff. Although this curve differs from that reported by Bradford *et al.* (*3*) for human smokers, data obtained on the smoking patterns of members of this laboratory varied considerably from individual to individual, and we are not prepared to designate this relationship as critical.

The more common devices for collecting particulate matter, *e.g.*, filters, cascade impactors, electrostatic precipitators, were felt to be poorly suited for the present problem since the amount of material to be trapped was relatively large and a nonoxidizing atmosphere was requisite. The system employed functions principally by impact and gravity deposition, aided by an increase in particle size by condensation and growth. Since cigarette smoke is a 2-phase system—particulate and vapor (*4*)—the collection train is well suited for trapping this material. A small amount of smoke escapes collection; the amount is estimated to be less than 0.1 percent of the total weight.

Equal numbers of 5 popular brands of 70 mm. blended American cigarettes are consumed. They are inserted in the holders of each manifold, the manifolds are connected *seriatim* to a supplementary source of low vacuum, the cigarettes are lit by means of a multi-jet gas burner, and the manifolds are fixed to the Y-tubes. The smoking operation is then initiated and continued until the median butt length is 2.5 cm. The spent cigarettes are removed, fresh cigarettes are inserted, and the cycle is repeated. The accumulated "tar" in the traps is periodically removed with the aid of acetone and is stored in full bottles at −20° C. until use.

### *Fractionation*

The smoke condensate-acetone mixture was distilled to 68° or to 36° C. per 220 mm.; distillate=AC, residue=B.

*A*) Residue B was cooled to 0° C. and washed with five 650 ml. portions of cold 2 *N* hydrochloric acid (quantity of acid for 1000 gm. of B) and with six 800 ml. portions of water. The combined acid extracts and water washes were centrifuged, and the sediment was washed with water and added to the acid-insoluble residue (D). The aqueous acid solution was designated A′B′.

*B*) In later runs B was cooled to 0° and extracted successively with two 900 ml. portions of cold water (per 1 kg. of B), 700 ml. of cold 12 percent hydrochloric acid, and 7–300 ml. portions of water; the mixture was agitated for 15 minutes after each addition. The combined extracts

were filtered through glass wool, and the residue was washed 3 times with 200 ml. portions of water which were added to the filtrate A′B′; the acid-insoluble substances constituted D. A′B′ was partially neutralized at 0° with 1 liter of 8 percent sodium hydroxide solution. The mixture was distilled to a temperature of 41° per 25 mm., 500 ml. of absolute ethanol was added, and the distillation repeated; this procedure was repeated twice again. To the residue was added 400 ml. of methanol, and the *p*H of the solution was adjusted to 8 with 10 percent methanolic sodium hydroxide. After removal of the solvent *in vacuo*, the material was extracted 3 times with acetone, using 800, 90, and 90 gm. successively. Vacuum distillation of the solvent from the extract gave AB. The residue was extracted successively with several other solvents to separate the organic material from the sodium chloride residue; evaporation of the solvents gave ABI.

D was distilled to 103° per 1.5 mm., giving a 2-phase distillate—E; the aqueous phase was extracted with ether, the solvent was removed, and the residue was added to the organic layer to constitute EA. The distillation residue, F, was extracted with petroleum ether (30–60°) using successively a 3100 ml. portion, a 620 ml. portion, two 620 ml. portions, and two 200 ml. portions. The residue, J′, was extracted with 910 ml. of acetone; the acetone-insoluble material was designated JJ and the acetone-soluble material, J.

The combined petroleum ether extracts were equilibrated with 670 ml. of 60 percent aqueous methanol, twice with 420 ml. portions of 80 percent aqueous methanol, and 5 times with 420 ml. measures of 92 percent aqueous methanol. Vacuum distillation of the solvent from the combined aqueous methanol extracts gave K. Distillation of the solvent from the petroleum ether layer gave M′, which was extracted with 410 ml. of acetone. The residue was called MM; the solvent-freed extract was named M.

The relative weights of the fractions expressed as percentages of F were K, 15.8; M, 33.6; MM, 11.3; J, 26.6; and JJ, 12.7. The values derived by using B as the basis of calculation are less meaningful, since the tar contains an appreciable quantity of water which is eliminated during the work-up, and the distribution is thus based on a hypothetical anhydrous tar; since fractions A′ and EA are not derived from F, these values are also cited: A′, 51; EA, 7; K, 4; M, 14; MM, 5; J, 12; JJ, 6. The values for all fractions except A′ are based on either 9 or 10 runs; the A′ value is based on 5 runs.

### *Preparation of* n-*Hentriacontane*

*16-Hentriacontanone.*—Synthesized by the method of Kipping (*5*) in 41 percent yield.

*Margaroyl chloride* (6).—To a solution of 10 gm. (36 *M*) of margaric acid in 36 ml. of benzene was added 8.4 gm. of phosphorus pentachloride over a 2-hour period. After refluxing for 1 hour, the mixture was distilled and the material boiling at 139 to 150° C. per 0.5 mm. was collected.

This was redistilled and 8.6 gm. (81% yield) of product with a b.p. of 140 to 148° per 0.5 mm. was obtained; lit. b.p. 139 to 140° per 0.4 mm. (*7*).

*15-Hentriacontanone.*—The ether was removed under vacuum from the Grignard reagent obtained from 12 gm. (43 m*M*) of 1-bromotetradecane and 1.07 gm. (44 m*M*) of magnesium after the addition of 4.6 gm. (25 m*M*) of cadmium chloride; 30 ml. of benzene was added, followed by 8.4 gm. (29 m*M*) of margaroyl chloride in 25 ml. of benzene. The mixture was refluxed for 2 hours, cooled, stirred with 60 ml. of 2.5 *N* hydrochloric acid, and filtered. The organic layer was washed with 150 ml. of 10 percent sodium carbonate solution and twice with water; after distillation of the benzene, the residue was recrystallized from ethanol to give 11.6 gm. (75%) of colorless ketone melting at 71.6 to 74.3° C. The m.p. was raised to 77.3 to 79.1° by chromatography on alumina and 2 additional recrystallizations from ethanol. *Analysis: Calculated for* $C_{31}H_{62}O$: C, 82.6; H, 13.9. *Found:* C, 81.4, 82.0; H, 13.3, 13.7.

n-*Hentriacontane.*—A mixture of 1.1 gm. of ketone (15- or 16-hentriacontanone), 17.0 ml. of 70 percent hydrazine hydrate, and 35 ml. of diethylene glycol was maintained at 110° C. for 1 hour; 6.5 gm. of potassium hydroxide was then added, and the mixture was refluxed for 8 hours at 185°. The acidified product was extracted twice with 200 ml. portions of benzene, and the residue resulting from the removal of the solvent was chromatographed on 200 gm. of alumina. The hydrocarbon was eluted with 300 ml. of petroleum ether (30–60°), the solvent was removed, and the residue was recrystallized from absolute ethanol. The 15-ketone gave a 49 percent yield (0.52 gm.) of hydrocarbon, m.p. 64.0 to 66.4°; the 16-ketone gave a 39 percent yield of product melting at 62.3 to 65.6°; both of these preparations absorbed faintly in the carbonyl region of the infrared spectrum. Purification was effected by chromatography on a mixture of 100 gm. of activated carbon (Darco) and 100 gm. of Celite 535 followed by ethanol recrystallization to give a colorless hydrocarbon, m.p. 68.5 to 69.2°; lit. m.p. 68.4 to 69.0° (*8*). *Analysis: Calculated for* $C_{31}H_{64}$: C, 85.2; H, 14.8. *Found:* C, 85.2; H, 14.8.

### *Fraction M′*

#### *Isolation of Paraffin Hydrocarbons*

A 40 gm. sample of M′ was chromatographed on 1 kg. of Merck activated alumina; 19.8 gm. of waxy orange material was eluted by 2 liters of petroleum ether (30–60° C.) and melted at 53 to 63°. A 7.7 gm. sample of this mixture in 15 ml. of carbon tetrachloride was introduced onto a chromatographic column composed of 520 gm. of a 1:1 mixture of activated carbon (Darco) and Celite 535 that had been wetted with ethanol. A series of solvents of graded polarity were employed as eluting agents.

*Identification of* n-*hentriacontane.*—After the removal of the more polar constituents with ethanol-carbon tetrachloride mixtures, a series of fractions was obtained with benzene-carbon tetrachloride mixtures whose infrared spectra were identical and apparently the same as that

of synthetic *n*-hentriacontane. The combined fractions (wt. 4.3 gm.) were recrystallized 8 times from ethanol-benzene to give colorless microcrystals, m.p. 69.9 to 71.1°, which did not depress the m.p. of synthetic *n*-hentriacontane, and which had an infrared spectrum indistinguishable from that of this compound. *Analysis: Calculated for* $C_{31}H_{64}$: C, 85.2; H, 14.8. *Calculated for* $C_{33}H_{68}$: C, 85.4; H, 14.7. *Found:* C, 85.3; H, 14.7.

The traces were obtained with a Norelco Diffractometer. The traces between 26 and 1.5° C. of the synthetic hentriacontane and the isolated hydrocarbon were both typical patterns of normal chain hydrocarbons. The long spacing of the sample from smoke was 43.0 ± 0.2 Å; the synthetic $C_{31}H_{64}$ had a spacing of 42.1 Å (the precision was poorer in this case owing to the small size of the sample). Kreger (*9*) lists the longest spacing of the $C_{31}$ hydrocarbon as 41.4 Å and that of the $C_{33}$ as 43.9 Å. The 43.0 Å value would be in accord with that expected for a mixture of the $C_{31}$ and $C_{33}$ hydrocarbons.

### *Sulfuric Acid Extraction*

A solution of 128.6 gm. M and 82.2 gm. of MM (M+MM=M′) in 3 liters of petroleum ether (30–60°) was extracted with sulfuric acid repeatedly until the acid layer was a light straw color. The acid-insoluble material was designated (A).

The combined acid extracts were poured onto excess ice and the resultant mixture was extracted with benzene. Removal of the solvent gave 183 gm. of (B). To (C), the aqueous residue, were added sodium chloride and sodium acetate to saturation. No sulfonic acid was obtained.

*Search for benzo*[a]*pyrene.*—(B) was dissolved in 1 liter of benzene and chromatographed on 2700 gm. of Davison silica gel, grade 12. A graded series of eluting solvents was employed and 20 cuts were taken. Each cut was examined in solution in a Beckman quartz cuvette and irradiated from above with ultraviolet light from a Hanovia "Inspectolite" lamp. The fluorescence was inspected by means of a Bausch and Lomb Bunsen spectroscope. It was found that the characteristic fluorescence bands of benzo[*a*]pyrene could be readily detected when a 0.001 percent solution of that hydrocarbon in benzene was employed.

Varying amounts of 0.01, 0.001, and 0.0001 percent benzo[*a*]pyrene solutions were spotted on Whatman No. 4 filter paper and developed by the descending technique, using a cyclohexane:*N*,*N*-dimethylformamide 3:2 solvent mixture. The limit of visibility of the fluorescence of the spots under ultraviolet light was found to be 6λ of a 0.0001 percent solution (equivalent to 0.006 μg. of hydrocarbon). The $R_f$ values were 0.89. A spot which fluoresced strongly enough to enable the characteristic bands to be distinguished with the spectroscope mentioned previously was obtained from 10λ of a 0.01 percent solution.

The several chromatographic cuts of the sulfuric acid extract described in the preceding experiment, together with M′, K, J, whole tar, and a benzene extract of JJ, were chromatographed under the same conditions for 4 hours. No spot was found with the same $R_f$ value and fluorescence

as that of a reference sample of benzo[*a*]pyrene. These materials were run on 5 sheets; the average $R_f$ value of benzo[*a*]pyrene was 0.79.

### *Sublimation*

A sublimation apparatus equipped with a dry ice-acetone cold finger containing 15 gm. of M′ was heated at continually increasing temperatures at 0.5 mm. *See* table 1.

TABLE 1.—*Sublimation of M′*

| Fraction | Temperature | Time | Weight |
|---|---|---|---|
| | | *(hours)* | *(mg.)* |
| 1 | 85–90° | 5 | 9. 7 |
| 2 | 90–102 | 5 | 26. 3 |
| 3 | 102–130 | 2 | 10. 7 |
| 4 | 130–135 | 4 | 23. 7 |
| 5 | 135–142 | 3. 5 | 16. 8 |
| 6 | 142–143 | 4 | 13. 9 |
| 7 | 143–145 | 3 | 12. 6 |
| 8 | 145–148 | 4 | 21. 8 |
| 9 | 148–151 | 1 | 10. 2 |
| 10 | 151–155 | 1 | 13. 1 |
| 11 | 155–158 | 1 | 16. 3 |
| 12 | 158–160 | 1 | 16. 7 |
| 13 | 160–162 | 1 | 17. 8 |
| 14 | 162–165 | 1 | 16. 2 |
| 15 | 165–168 | 1 | 17. 3 |

### *Chromatography of Fraction M*

Fraction M (54.2 gm.) was chromatographed on 4400 gm. of Davison silica gel, grade 12, as shown in table 2.

TABLE 2.—*Chromatography of M*

| Fraction | Solvent | Volume *(ml.)* | Wt. of fraction *(gm.)* |
|---|---|---|---|
| 1 | Petroleum ether | 800 | 5. 38 |
| 2 | Petroleum ether | 1900 | 1. 42 |
| 3 | Petroleum ether | 4000 | 1. 24 |
| | $C_6H_6$ in petroleum ether | | |
| 4 | 16% | 4800 | 2. 16 |
| 5 | 36% | 4000 | 4. 17 |
| 6 | 85% | 4000 | 5. 61 |
| 7 | 97% | 3000 | 1. 92 |
| 8 | Benzene | 6000 | 3. 15 |
| | Ether in $C_6H_6$ | | |
| 9 | 5% | 7000 | 1. 92 |
| 10 | 5% | 2000 | 1. 61 |
| | Acetone in $C_6H_6$ | | |
| 11 | 15% | 4000 | 19. 22 |
| 12 | 17% | 3000 | 1. 59 |
| 13 | 36% | 7000 | 2. 31 |
| 14 | 45% | 4000 | 0. 74 |
| 15 | Acetone | 3000 | 0. 26 |
| | Methanol in acetone | | |
| 16 | 35% | 4000 | 0. 38 |
| 17 | 35% | 4000 | 0. 81 |
| 18 | Acetic acid | 2500 | 0. 61 |

### *Chromatography of Fraction MM*

A solution of 19.4 gm. of MM in 100 ml. of petroleum ether (30–60°) was chromatographed on 895 gm. of Davison silica gel, grade 12. *See* table 3.

TABLE 3.—*Chromatography of MM*

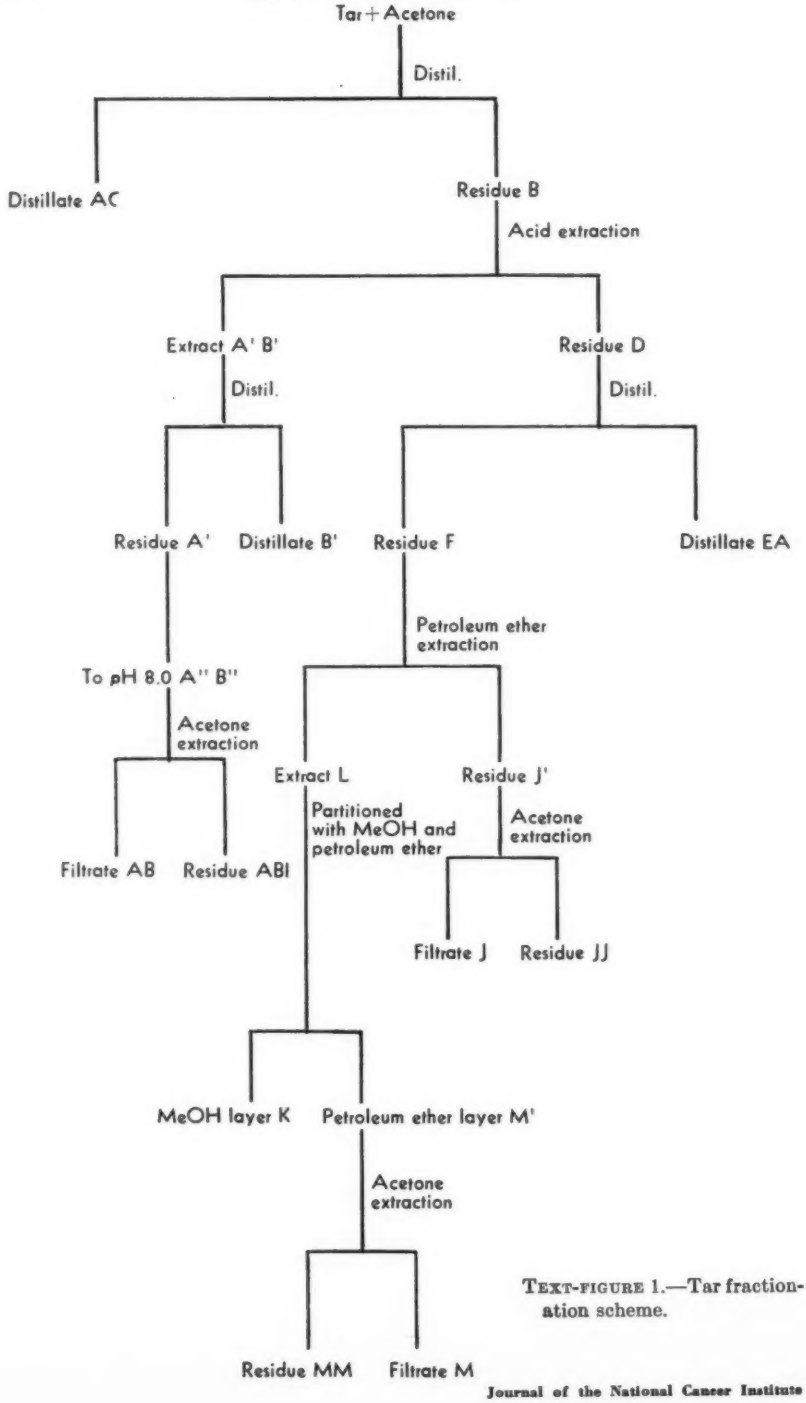
| Fraction | Solvent | Volume (*ml.*) | Wt. of fraction (*gm.*) |
|---|---|---|---|
| 1 | Petroleum ether | 300 | 2. 99 |
| 2 | Petroleum ether | 400 | 6. 02 |
| 3 | Petroleum ether | 3800 | 1. 22 |
| | $C_6H_6$ in petroleum ether | | |
| 4 | 15% | 3200 | 1. 18 |
| 5 | 50% | 1800 | 0. 80 |
| 6 | 92% | 1800 | 0. 69 |
| 7 | Benzene | 1600 | 0. 38 |
| 8 | Benzene | 2500 | 0. 61 |
| | Acetone in $C_6H_6$ | | |
| 9 | 2% | 1800 | 1. 43 |
| 10 | 3% | 1600 | 0. 35 |
| 11 | 4% | 1800 | 1. 22 |
| 12 | 5% | 800 | 0. 78 |
| 13 | 20% | 1600 | 0. 51 |
| 14 | 20% | 1800 | 0. 19 |
| 15 | 24% | 2000 | 0. 13 |
| 16 | 28% | 2000 | 0. 70 |
| 17 | 30% | 2000 | 0. 60 |
| 18 | 34% | 1800 | 0. 20 |
| 19 | Acetone | 2400 | 0. 43 |
| 20 | Chloroform | 1000 | 0. 28 |
| 21 | Methanol | 3000 | 0. 14 |
| 22 | Acetic acid | 1800 | 0. 21 |

### *Biological Tests*

Fractions B, D, F, AB, EA, J, JJ, K, M, and MM were tested for their ability to cause disappearance of sebaceous glands in mice, a reaction which has shown a correlation with carcinogenicity (*10*); B, D, F, K, M, and J gave positive tests. These same fractions, excluding F, were also used in long-term skin-painting tests on mice; B, D, M, and K yielded papillomas. K, J, M, and B were assayed by long-term painting of rabbits; B, K, and M produced papillomas. Full details of the biological-testing program will be reported by Drs. W. E. Smith, L. Orris, and N. S. Cooper in another paper.

## Results and Discussion

A large-capacity smoking machine was constructed to provide smoke condensate for both chemical and biological studies. To facilitate comparison of our results with those obtained in other laboratories, it was decided to conform as nearly as possible to the smoking pattern of Bradford, Harlan, and Hanmer (*3*), which has been generally adopted by the American cigarette industry and which is said to be a good approximation of human smoking habits. This scheme requires a puff of 35 ml. volume and of 2-second duration, with a 58-second interval between puffs.

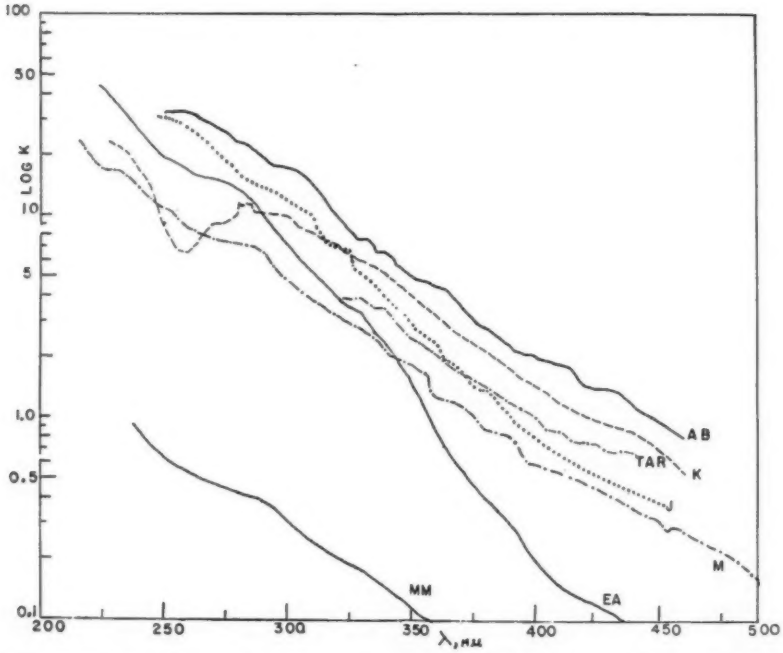



TEXT-FIGURE 1.—Tar fractionation scheme.

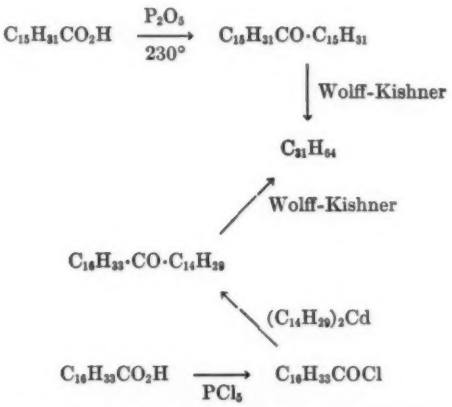
The acetone-smoke condensate mixture was separated into 8 primary fractions as outlined in text-figure 1. Chromatography was deemed inadvisable at this stage because of the quantity of material involved, although pilot experiments indicated that a useful separation was achieved thereby. Extraction of D with sodium bicarbonate solution to remove strong acids was discontinued because the amount of material thus removed was small; and extraction of D with sodium hydroxide solution to remove acidic material was deemed inadvisable, since the alkali-insoluble material had an infrared absorption spectrum indicative of the presence of carbonyl groups.

The ultraviolet absorption spectra of the smoke condensate ("tar") and of the primary fractions other than JJ are given in text-figure 2. The general lack of detail in the curves suggests that each fraction is a complex mixture. Of particular interest is the low over-all absorption of MM, which would be consistent with a high content of aliphatic hydrocarbon. Hentriacontane had been reported in tobacco smoke (*11–13*), and we were able to isolate an aliphatic hydrocarbon with the physical properties expected for this compound from both M′ and MM by chromatography on either alumina or silica gel, followed by chromatography on charcoal-Celite; this material (crude m.p. 64.5–66.0° C.) represents about 55 percent by weight of MM. The *normal* structure of this "hentriacontane" had not been demonstrated by previous investigators, and a synthetic sample was therefore prepared, for comparison, by the procedure of Kipping (*5*) from palmitic acid and, by a related synthesis, from margaric acid as shown in text-figure 3. Although a mixed melting point of the isolated and synthetic samples showed no depression, this cannot be taken as proof of identity with such compounds. The infrared spectra of the 2 materials were virtually identical, which was a strong indication that the isolated hydrocarbon was *normal*. The X-ray diffraction pattern of the isolated "hentriacontane" was markedly suggestive of a mixture of the normal $C_{31}$ compound with *n*-tritriacontane. It seems likely that the "hentriacontane" reported by previous workers also contained the higher paraffin hydrocarbon. Thorpe and Holmes (*14*) isolated 2 hydrocarbons from tobacco leaf to which they assigned the structures hentriacontane and heptacosane. Their data for the compound, said to be $C_{27}$, are not internally consistent, and in view of the results reported above, they warrant reinvestigation, although we do not dispute the presence of hydrocarbons of smaller chain length than $C_{31}$ in tobacco leaf (and, indeed, have some evidence for their existence in smoke).

The discrepancies in the literature concerning the occurrence of benzo[*a*]pyrene in tobacco smoke [(*2*), p. 19] prompted us to search for this compound in our "tars." A sample of M′, the fraction which solubility considerations suggested as the most likely locus of polynuclear hydrocarbons, was extracted with sulfuric acid following Berenblum's (*15*) procedure for the isolation of benzo[*a*]pyrene. The acid-soluble, unsulfonated material was chromatographed on silica gel and the 20 chromatographic cuts were inspected for the characteristic benzo[*a*]pyrene fluor-




TEXT-FIGURE 2.—M and MM in cyclohexane; tar (B) in acetone; EA, K, J, AB in 95 percent ethanol.




TEXT-FIGURE 3.—Synthesis of *n*-hentriacontane.

escence spectrum (*16*) under ultraviolet light. Although the fluorescence bands of a 0.001 percent solution of benzo[*a*]pyrene in benzene could be distinguished with the equipment used, no such bands were visible in any of these cuts. However, the possibility of quenching of fluorescence in solutions of these complex mixtures militates against placing too much

weight on these data; in general the fluorescence was not intense enough to give any definitive spectrum.

The several chromatographic fractions were then chromatographed on paper using a 3:2 cyclohexane:*N*,*N*-dimethylformamide solvent mixture in the descending technique. It had been determined that as little as 0.006 μg. of benzo[*a*]pyrene could be detected on paper by its fluorescence under ultraviolet light; no spot was found with the same $R_f$ value as a reference sample of the hydrocarbon. Similar negative results were obtained when fractions M′, K, J, a benzene extract of JJ, and whole tar were chromatographed on paper.

The ultraviolet absorption spectra of extended series of chromatographic fractions of M and MM and of a series of sublimates of M′ showed no characteristic benzo[*a*]pyrene bands.

These results are in disagreement with those of Cooper, Lindsey, and Waller (*17*) but agree with those of several other investigators [(*12*), p. 19]. A possible explanation of the discrepancy in results may lie in the ease with which benzo[*a*]pyrene undergoes photooxidation (*18–20*); during a relatively lengthy fractionation scheme such as our own, it is conceivable that if the hydrocarbon were present, it might be destroyed during the work-up; however, this argument does not apply to the paper-chromatographic work on whole tar. Differences in the cigarettes used (both chemical and physical), in the smoking procedures, and in the humidity of the environment, may also be significant in this regard. The disagreement on the presence of benzo[*a*]pyrene in cigarette smoke would seem to be of little biological significance, since the amount reported to be present is below the threshold concentration for induction of skin cancer in mice (*21*); and we feel that this fact also minimizes the importance of the probable distribution of the hydrocarbon between 2 fractions (*e.g.*, M and K) in certain of the procedures described. It should be noted that: 1) even if the material were partitioned equally between 2 fractions, *e.g.*, K and M, benzo[*a*]pyrene in the concentration reported by Cooper *et al.* (*17*) should have been detected by the paper-chromatographic method unless additional loss were suffered owing to 1 of the factors mentioned above; 2) fractions K and M each elicited tumors in skin-painting experiments (*see* Experimental); and 3) this discussion is concerned with only the 1 polynuclear hydrocarbon benzo[*a*]pyrene and does not imply that there are no polynuclears present in cigarette smoke; we have observed peaks in the ultraviolet absorption spectra of certain smoke-condensate fractions which are highly suggestive of polynuclear-hydrocarbon absorption, but we do not feel that these can be taken as positive proof of identity.

## Summary

1) A large-capacity cigarette-smoking machine has been devised.

2) Cigarette-smoke condensate has been separated into 8 fractions.

3) Indicative proof of the presence of *n*-hentriacontane and *n*-tritriacontane in the smoke has been obtained.

4) Analyses for benzo[*a*]pyrene were negative.

## References


(*1*) WYNDER, E. L., GRAHAM, E. A., and CRONINGER, A. B.: Experimental production of carcinoma with cigarette tar. Cancer Res. 13: 855–864, 1953.

(*2*) KOSAK, A. I.: Chemistry. *In* The Biological Effects of Tobacco (Wynder, E. L., ed.). Boston, Little, Brown & Co., 1955, pp. 1–34.

(*3*) BRADFORD, J. A., HARLAN, W. R., and HANMER, H. R.: Nature of cigarette smoke. Indust. & Eng. Chem. 28: 836–839, 1936.

(*4*) WENUSCH, A.: Der Tabakrauch. Bremen, Geist, 1939.

(*5*) KIPPING, F. S.: Action of phosphoric anhydride on fatty acids. Part 2. J. Chem. Soc. 57: 980–988, 1890.

(*6*) SHERK, K. W., AUGUR, M. V., and SOFFER, M. D.: High molecular weight hydrocarbons I. Eicosane, 1-phenyleicosane, 3-ethyloctadecane. J. Am. Chem. Soc. 67: 2239–2240, 1945.

(*7*) FIERZ-DAVID, H. E., and KUSTER, W.: Homologous series of acylated azo dyes from *o* and *p*-acylaminophenols and 1,7-acylaminonaphthols. Helvet. chim. acta 22: 82–112, 1939.

(*8*) GASCARD, A., and DAMOY, G.: Alcohols and hydrocarbons in beeswax. Compt. rend. Acad. d. sc. 177: 1442–1443, 1923.

(*9*) KREGER, D. R.: Investigations in the biological fields. *In* Selected Topics in X-ray Crystallography (Bouman, J. ed.). Amsterdam, North Holland, 1951, p. 316.

(*10*) SMITH, W. E., COOPER, N. S., and WYNDER, E. L.: Biological tests of fractions derived from cigarette smoke condensates. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 45, 1954.

(*11*) SCHÜRCH, O., and WINTERSTEIN, A.: Tobacco and cancer. Ztschr. Krebsforsch. 42: 76–92, 1935.

(*12*) WENUSCH, A.: Occurrence of higher hydrocarbons in tobacco smoke. Biochem. Ztschr. 273: 178–179, 1934.

(*13*) ———: Resins of cigar smoke. Ztschr. Untersuch. Lebensmitt. 73: 189–191, 1937.

(*14*) THORPE, T. E., and HOLMES, J.: The occurrence of paraffins in the leaf of tobacco. J. Chem. Soc. 79: 982–986, 1901.

(*15*) BERENBLUM, I.: 3,4-Benzpyrene from coal tar. Nature, London, 156: 601, 1945.

(*16*) HIEGER, I.: Spectra of cancer-producing tars and oils and of related substances. Biochem. J. 24: 505–511, 1930.

(*17*) COOPER, R. L., LINDSEY, A. J., and WALLER, R. E.: The presence of 3,4-benzpyrene in cigarette smoke. Chem. and Indust. 46: 1418, 1954.

(*18*) COOK, J. W., MARTIN, R., and ROE, E. M. F.: Photo-oxides of carcinogenic hydrocarbons. Nature, London, 143: 1020, 1939.

(*19*) ALLSOPP, C. B.: Photo-oxides of carcinogenic hydrocarbons. Nature, London, 145: 303, 1940.

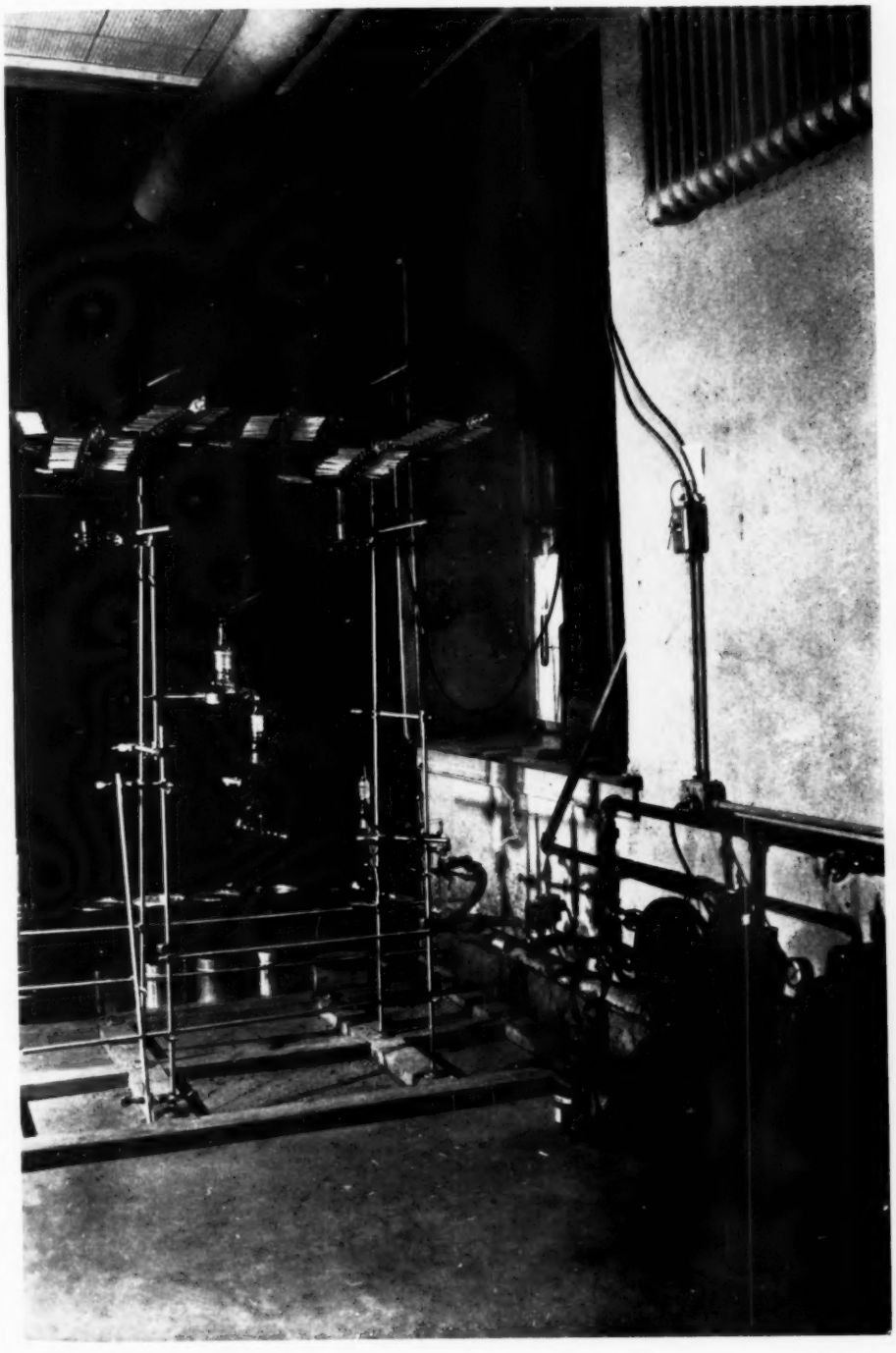
(*20*) ———: A water-soluble carcinogen derived from 3,4 benzpyrene. Nature, London, 150: 548–549, 1942.

(*21*) WYNDER, E. L.: Personal communication; *cf.* Chem. & Eng. News 34: 2242, 1956.

PLATE

PLATE 25

Smoking machine.





393045—56——14

# Influence of Amethopterin on Tumor Growth and Liver Glutathione Levels of Mice Bearing Lymphocytic Leukemias [1, 2]

BERNARD SHACTER, *and* L. W. LAW, *National Cancer Institute,*[3] *Bethesda, Maryland*

Numerous investigations have demonstrated that glutathione (GSH) plays an important, although as yet not clearly defined, role in cell division (*1*). The possibility of an altered metabolism of GSH in neoplastic disease has therefore been considered frequently (*2*).

In human leukemia, the observation has been made (*3,4*) and disputed (*5*) that leukemic cells have a higher GSH content than normal leukocytes. The relationship of GSH to the leukemic process was explored further in the present experiments through determination of the influence of a growth-inhibitory agent on GSH levels of tumor cells and livers of mice bearing experimental leukemias.

Glutathione and ascorbic acid appear intimately associated in cell metabolism, and stresses which affect the cellular concentration of one often affect the other (*6,7*). Moreover, ascorbic acid like GSH appears to play a role in cell division (*8,9*). Ascorbic acid assays were therefore also included in the present work.

## Materials and Methods

Unless otherwise indicated, the experiments were carried out with (C3Hf × AKR)$F_1$ hybrid mice, approximately 3 months old, bearing ascitic growths of the lymphocytic leukemia L4946, of strain AKR origin.[4] Tumors grow rapidly following the implanting of about 1 million cells intraperitoneally, attaining maximum growth on the 7th post-transplant day. Untreated mice are usually dead on the 8th day. Amethopterin (4-amino-$N^{10}$-methyl-pteroylglutamic acid) has a profound effect in inhibiting growth of this neoplasm. When administered at a dose level of 3 mg. per kg. every other day, beginning with the day following transplantation, survival may be extended to over 30 days.[5]

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[1] Received for publication June 12, 1956.

[2] Presented in part at a meeting of the American Association for Cancer Research, Atlantic City, N. J., April 1956 (Proc. Am. Assoc. Cancer Res. 2: 146, 1956).

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] The specific sublines used were: C3Hf/Lw and AKR/Lw. These $F_1$ mice were produced in our laboratory. Line B of L4946 transfer generations 55 to 73 was used.

[5] Unpublished data.

Change in packed volume of ascitic cells was used as a measure of tumor proliferation. Mice fasted overnight were killed by cervical dislocation, and the peritoneal fluid was drawn off as completely as possible in a syringe, usually after dilution with Locke's solution. After centrifugation, the supernatant fluid was discarded and the packed cells were allowed to drain. A measured volume of Locke's solution was then added to the cells, and the cells were uniformly suspended. The packed-cell volume of the suspension was determined by centrifugation of an aliquot in a Wintrobe hematocrit tube, and from this the total volume of ascites cells removed could be readily calculated. The remaining suspension was used for determination of the GSH content per ml. of packed cells.

Livers were excised as rapidly as possible after sacrifice, weighed, and homogenized in cold distilled water to give a 10 percent homogenate. Aliquots were then added to metaphosphoric acid for GSH determination by the nitroprusside procedure of Grunert and Phillips (*10*), or to trichloroacetic acid for ascorbic acid assay by the Schaffert and Kingsley (*11*) modification of the dinitrophenylhydrazine method of Roe and Kuether (*12*). Results were calculated on the basis of wet weight of liver.

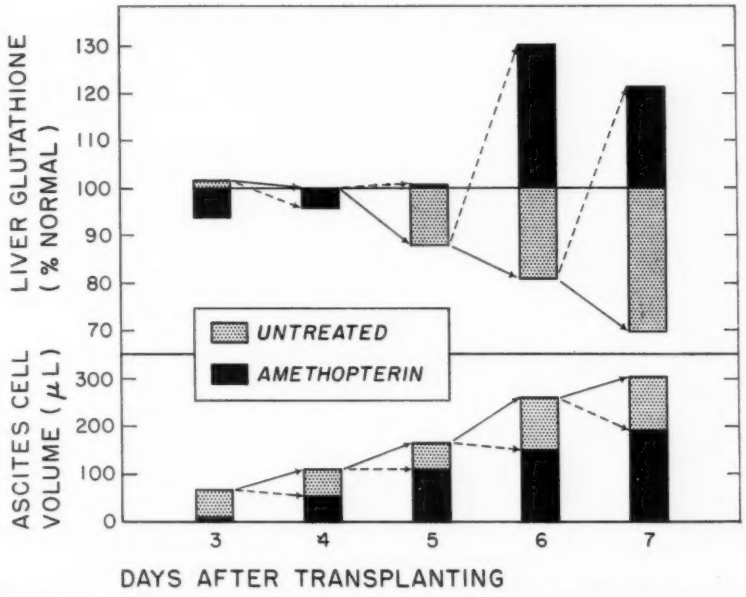
## Results

Several preliminary experiments were carried out to determine the effect of amethopterin on GSH levels of the leukemic cells. Mice bearing 6-day-old growths of the tumor were divided into 2 groups. Treated mice received 15 mg. per kg. of amethopterin intraperitoneally at about 20 hours, and again 2 hours prior to sacrifice. Untreated mice were given equivalent volumes of Locke's solution. No differences were found in GSH levels of ascitic-tumor cells of the 2 groups; pronounced differences, however, were evident in liver GSH levels. The tripeptide concentration, markedly lowered in untreated mice, was raised appreciably above normal after treatment with amethopterin. Further investigation demonstrated that a single injection of 3 mg. per kg. of amethopterin, administered 18 to 20 hours prior to sacrifice, was as effective as the 2 larger doses in bringing about the observed result. Pteroylglutamic acid, on the other hand, was completely ineffective in influencing liver GSH levels of tumor-bearing mice.

Liver GSH determinations carried out at graded intervals, beginning 3 hours after administration of amethopterin to mice bearing 6-day-old tumors, showed that the increase in liver GSH became apparent at about 12 hours and was strongly evident at 15 and 18 hours after injection. Amethopterin had no effect on liver GSH levels of normal mice.

With the observation that the liver GSH levels of mice bearing large growths of L4946 were lowered and that amethopterin reversed this effect, experiments were next carried out to study the relationship between tumor growth and liver GSH and ascorbic acid levels, on the one hand, and the influence of amethopterin, when administered at successive stages of growth, on the other. The tumor was transplanted to a large group of

female mice on day zero.[6] On the second post-transplant day a portion of the mice received 3 mg. per kg. of amethopterin and an equal number were given an equivalent volume of Locke's solution. The mice were killed on the 3rd post-transplant day, 18 to 20 hours after treatment. The same procedure was followed on the 4 succeeding days. Non-tumor-bearing mice served as normal controls for the liver assays. Results are shown in text-figure 1 and table 1.




TEXT-FIGURE 1.—Relationship between tumor growth and liver glutathione levels of untreated and amethopterin-treated mice. *Broken arrows* indicate direction of change for amethopterin-treated mice; *solid arrows* indicate trend in untreated mice. Values shown are means for 12 mice in each group on days 3, 4, and 5, and 8 mice on days 6 and 7. Liver glutathione concentrations of 48 normal mice averaged 502 $\mu M$ per 100 gm.

An inhibitory effect of amethopterin on tumor growth was readily detectable in animals treated 2 days after transplantation. Following single treatment with amethopterin on succeeding days, tumor growth was seemingly completely halted; the volume of cells, however, did not appear to decrease appreciably.

An effect of tumor growth on liver GSH did not become evident until after the 4th post-transplant day, when the levels began decreasing. The downward trend continued for the remaining 2 days of the experiment.

[6] There appeared to be a sex difference in the growth of the tumor. Six days after transplantation, the ascitic-cell volume of female mice was invariably greater than that of male mice, and liver GSH levels were lower. Moreover, amethopterin had a somewhat greater effect in raising liver GSH levels of female than of male mice. Females were therefore used in later experiments.

Amethopterin had no distinct effect on liver GSH levels during the early stages of tumor growth. When administered on the 5th day of growth, at which time the GSH content had become somewhat lowered, there was a marked rise above normal in liver GSH. The same effect was evident following treatment on the 6th day. Maximum differences between liver GSH concentrations of treated and untreated mice were usually found on the 7th post-transplant day.

In contrast to the changes observed for GSH, liver ascorbic acid levels of untreated, as well as amethopterin-treated, mice remained essentially unchanged during tumor growth. Liver weight, expressed as percent total-body weight, remained normal until the 7th post-transplant day, increasing about 20 percent for both control and experimental groups at that time.

TABLE 1.—*Effect of tumor growth and amethopterin on liver weight and ascorbic acid**

| Treatment | Day after transplantation | Liver ascorbic acid | Liver weight |
|---|---|---|---|
| Control | 3 | 98 (8) | 104 (12) |
| | 4 | 97 (8) | 97 (12) |
| | 5 | 93 (8) | 104 (12) |
| | 6 | 95 (8) | 105 ( 8) |
| | 7 | 87 (8) | 121 ( 8) |
| Amethopterin | 3 | 101 (8) | 100 (12) |
| | 4 | 100 (8) | 98 (12) |
| | 5 | 101 (8) | 103 (12) |
| | 6 | 104 (8) | 101 ( 8) |
| | 7 | 102 (8) | 118 ( 8) |

*All values expressed as percent normal. Numbers in parentheses indicate the number of animals. Average values for normal control animals were as follows: liver ascorbic acid, 203 $\mu M$ per 100 gm. (40 mice); liver weight, 5.22 percent body weight (51 mice).

If it is considered that the decreases in liver GSH of untreated tumor-bearing mice were related to the growth of the tumor, and that the elevations which followed administration of amethopterin were events secondary to the inhibition of tumor growth, then it might be expected that the agent would decrease rather than increase liver GSH levels if administered to mice bearing tumors dependent on it for optimal growth. This possibility was tested in mice bearing leukemia L1210/AM-D, a tumor dependent on amethopterin for optimal growth (*13*). Mice bearing the tumor were divided into experimental and control groups. The former received 3 mg. per kg. of amethopterin intraperitoneally every other day, beginning with the 1st day after transplantation; the latter were given equivalent volumes of Locke's solution. All mice were sacrificed on the 7th post-transplant day. As shown in table 2, liver GSH levels of amethopterin-treated mice decreased appreciably below both control and normal values.

TABLE 2.—*Effect of amethopterin on liver GSH of mice bearing L1210/AM-D ascitic tumors**

| Treatment | Liver GSH† | |
|---|---|---|
| | *μM/100 gm.* | *Percent normal* |
| Control | 508 (6) | 96 |
| Amethopterin | 316 (6) | 60 |

*Carried out with (C × DBA)F$_1$ mice produced in the NIH animal colony by crossings of the specific sublines B/A/L/B/CAnN and DBA/2JN, using line F of L1210/AM-D, transfer generations 55 to 70.

†Average results of 2 experiments on total number of mice indicated in parentheses. Mean liver GSH of 6 normal mice was 528 *μM* per 100 gm.

The influence of several other agents on liver GSH levels of mice bearing 6-day-old growths of L4946 is shown in table 3. Each experiment included a group of mice treated with amethopterin as the standard agent, as well as a control group of untreated tumor-bearing mice. Results are expressed as the percentage ratio of liver GSH in treated as compared to untreated mice.

TABLE 3.—*Influence of several compounds on liver glutathione levels of mice bearing the ascitic form of lymphocytic leukemia L4946*

| Experiment | Treatment* | No. of mice | Total dosage† | Route of injection | Liver GSH |
|---|---|---|---|---|---|
| | | | *Mg./kg.* | | *Percent control* |
| 1 | DL-Ethionine | 3 | 375 | I.P. | 150 |
| | Ethionine + amethopterin | 3 | 375 + 15 | I.P. | 260 |
| | Amethopterin | 3 | 15 | I.P. | 207 |
| 2 | 8-Azaguanine | 3 | 75 | S.C. | 90 |
| | 6-Mercaptopurine | 3 | 75 | S.C. | 126 |
| | 2,6-Diaminopurine | 3 | 75 | S.C. | 108 |
| | Amethopterin | 3 | 3 | I.P. | 207 |
| 3 | Azaserine | 3 | 27 | I.P. | 125 |
| | Azaserine + amethopterin | 3 | 27 + 15 | I.P. | 174 |
| | Amethopterin | 3 | 15 | I.P. | 185 |
| 4 | 6-Azauracil | 6 | 1000‡ | S.C. | 102 |
| | Amethopterin | 6 | 3 | S.C. | 164 |
| 5 | 4APP§ | 6 | 30‡ | S.C. | 176 |
| | Amethopterin | 6 | 3 | I.P. | 174 |

*Compounds were kindly supplied as follows: Amethopterin and 8-azaguanine, Lederle Laboratories Division, American Cyanamid Co.; 6-mercaptopurine and 2,6-diaminopurine, Wellcome Research Laboratories; 6-azauracil, Dr. Arnold D. Welch; 4APP, Dr. Roland K. Robins.

†Administered in a single injection 18 to 20 hours prior to sacrifice unless otherwise indicated.

‡In divided doses at 20 and 10 hours prior to sacrifice.

§4-Aminopyrazolo (3, 4-*d*) pyrimidine.

Ethionine slightly increased liver GSH levels of tumor-bearing mice and potentiated the action of amethopterin, thus paralleling its action on survival times of mice bearing the solid form of L4946 (*14,15*). 8-Azaguanine, 6-mercaptopurine, and 2,6-diaminopurine have no effect on

liver GSH, nor on survival time and tumor size of mice bearing the solid form of the tumor.[7] 4APP, which inhibits growth of L4946,[8] distinctly increased liver GSH levels.

In the action of only 1 compound did there appear to be a discrepancy between inhibition of the growth of the solid tumor and effect on liver GSH of mice bearing ascites growths. 6-Azauracil has been found by Hakala *et al.* (*16*) to inhibit growth of the solid form, while no effect was noted on liver GSH levels. Since the maintenance of adequate tissue levels is of major importance in the inhibitory action of 6-azauracil, the negative effects on liver GSH may perhaps be related to the dosage and timing of administration of the compound.

## Discussion

Decreases in the glutathione content of the liver have been observed following various experimental conditions, including total-body X irradiation (*17*), physical stresses (*18,19*), and nutritional deficiencies (*20–22*). While the over-all effect of decrease in liver GSH would therefore appear to be nonspecific, it seems likely that the mechanisms involved in bringing this about may differ.

The decrease in liver GSH which follows exposure to cold and restraint occurs very rapidly, while regeneration of GSH is a much slower process (*18*). Register (*23*) showed that the same changes in liver GSH could be elicited with epinephrine, and suggested a sympathoadrenal mechanism as one of the factors regulating GSH levels in the liver. Perhaps this may be the mechanism involved in bringing about liver GSH decreases following physical stress. The decrease in liver GSH associated with nutritional deficiencies, on the other hand, seems more likely to be the result of a diminished rate of synthesis.

In the present experiments, decreases in liver GSH did not become apparent until the tumors had reached a critical size. Moreover, there was marked and rapid overshooting of normal levels upon suppression of tumor growth. These data are interpreted as indicating an increased requirement for GSH from the liver during tumor growth, accompanied by an increased output by the liver. In the earlier stages of growth, the supply keeps up with the demand and there is no net change in liver GSH levels. As the tumor becomes sufficiently large, however, demand outstrips supply, and liver GSH levels begin to fall. The inhibition of growth brought about by amethopterin lessens the requirement for GSH, and the markedly increased output by the liver, previously masked by rapid utilization, is manifested by an elevation above normal levels.

Decreases in sulfhydryl levels of serum and plasma have previously been shown to occur in neoplastic disease (*24,25*) and following X irradiation and surgical trauma (*26*). It was suggested that the decreases were indicative of an increased rate of utilization of sulfhydryl groups during tissue proliferation, with the plasma serving as an immediate source of

[7] Unpublished data.
[8] Unpublished data of Dr. S. E. Reaume.

sulfhydryl groups for tissue requirements and being replenished in turn from some sulfhydryl source (*25*). It would be of interest to determine whether there is a relationship between decreases in plasma protein-bound sulfhydryl groups and liver GSH levels during tumor growth.

Amethopterin apparently has no effect on cellular GSH, as indicated by its inability to change GSH levels of either leukemic cells or livers of normal mice. Thus, although the experiments indicate increased utilization of GSH during growth of the leukemic cells, they shed no light on the question of an altered concentration of GSH in individual cells.

There appeared to be close correlation between the action of several chemotherapeutic agents on tumor size or survival time of mice bearing solid growths of L4946, on the one hand, and their effect on liver GSH levels of mice bearing ascitic growths of the tumor on the other. Measurement of their influence on liver GSH levels of tumor-bearing mice may therefore provide an auxiliary index of the efficiency of carcinostatic agents. The method may have particular application where the supply of agent is limited.

## Summary

The influence of tumor growth and of amethopterin administration on glutathione levels of liver and tumor cells was studied in mice bearing rapidly growing lymphocytic leukemias in ascitic form. Amethopterin, administered about 1 day prior to sacrifice, had no effect on glutathione levels of the leukemic cells. Growth of the tumor was accompanied, in the later stages, by a progressive fall in liver glutathione levels. When administered to mice bearing large growths of the tumor, amethopterin inhibited growth and produced a marked rise in liver glutathione above normal levels. It is suggested that there was an increased requirement for glutathione from the liver during growth of the tumor, accompanied by an increased output by the liver.

The influence of several chemotherapeutic agents on liver glutathione levels of mice bearing ascitic growths of L4946 paralleled their known influence on tumor size and survival time of mice bearing solid growths of the tumor.

## References

(*1*) Barron, E. S. G.: Thiol groups of biological importance. Advances Enzymol. 11: 201–266, 1951.

(*2*) Stern, K., and Willheim, R.: The Biochemistry of Malignant Tumors. Brooklyn, N. Y., Chem. Pub. Co., Inc., 1943, 951 pp.

(*3*) Contopoulos, A. N., and Anderson, H. H.: Sulfhydryl content of blood cells in dyscrasias. J. Lab. & Clin. Med. 36: 929–941, 1950.

(*4*) Green, R., and Martin S. P.: The nonprotein soluble sulfhydryl content of human leukocytes and erythrocytes in infection and leukemia. J. Lab. & Clin. Med. 45: 119–122, 1955.

(*5*) Hardin, B., Valentine, W. N., Folletff, J. H., and Lawrence, J. S.: Studies on the sulfhydryl content of human leukocytes and erythrocytes. Am. J. Med. Sc. 228: 73–82, 1954.

(*6*) Meiklejohn, A. P.: The physiology and biochemistry of ascorbic acid. Vitamins and Hormones 11: 61–96, 1953.

(7) Glutathione and ascorbic acid in liver necrosis. Nutrition Revs. 12: 177–178, 1954.

(8) Stern, H., and Timonen, S.: The position of the cell nucleus in pathways of hydrogen transfer: cytochrome c, flavoproteins, glutathione, and ascorbic acid. J. Gen. Physiol. 38: 41–52, 1954.

(9) Gol'dshteĭn, B. I., Gerasimova, V. V., and Kondrat'eva, L. G.: Influence of vitamin C on conversion of nucleic acids in animal tissues. Biokhimiya, Moscow, 19: 531–542, 1954.

(10) Grunert, R. R., and Phillips, P. H.: A modification of the nitroprusside method of analysis for glutathione. Arch. Biochem. 30: 217–225, 1951.

(11) Schaffert, R. R., and Kingsley, G. R.: A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. J. Biol. Chem. 212: 59–68, 1955.

(12) Roe, J. H., and Kuether, C. A.: The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147: 399–407, 1943.

(13) Law, L. W.: The phenomena of resistance and dependence in leukaemic cells. *In* Ciba Foundation Symposium on Leukaemia Research (Wolstenholme, G. E. W., and Cameron, M. P., eds.). London, J. & A. Churchill, Ltd., 1954, pp. 105–126.

(14) Skipper, H. E., Thomson, J. R., and Bell, M.: Attempts at dual blocking of biochemical events in cancer chemotherapy. Cancer Res. 14: 503–507, 1954.

(15) Morrison, S. S., and Higgins, G. M.: An attempt to "block" sources of methyl groups in the therapy of mouse leukemia. Cancer Res. 16: 292–299, 1956.

(16) Hakala, M. T., Law, L. W., and Welch, A. D.: Inhibitory activity of 6-azauracil, 6-uracil methyl sulfone, and related compounds on the growth of mouse lymphomas and sarcoma 180. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 113, 1956.

(17) Peterson, R. D., Beatty, C. H., and West, E. S.: Effect of total body X-irradiation on glutathione levels in rats. Proc. Soc. Exper. Biol & Med. 77: 747–749, 1951.

(18) Register, U. D., and Bartlett, R. G., Jr.: Regeneration of tissue nonprotein sulfhydryl compounds in rats after exposure to cold and restraint. Proc. Soc. Exper. Biol. & Med. 86: 836–838, 1954.

(19) Beck, L. V., Linkenheimer, W. H., and Marraccini, A.: Effects of tumbling trauma, scalding and hemorrhage on rat tissue non-protein sulfhydryl. Proc. Soc. Exper. Biol. & Med. 86: 823–827, 1954.

(20) Leaf, G., and Neuberger, A.: The effect of diet on the glutathione content of the liver. Biochem J. 41: 280–287, 1947.

(21) Grunert, R. R., and Phillips, P. H.: Uric acid diabetes in the rat. Proc. Soc. Exper. Biol. & Med. 76: 642–645, 1951.

(22) Register, U. D.: Effect of vitamin $B_{12}$ on liver and blood non-protein sulfhydryl compounds. J. Biol. Chem. 206: 705–709, 1954.

(23) Register, U. D., and Bartlett, R. G., Jr.: Relationship of adrenalin to tissue sulfhydryl compounds. Science 120: 109–110, 1954.

(24) Schoenbach, E. B., Weissman, N., and Armistead, E. B.: The determination of sulfhydryl groups in serum. II. Protein alterations associated with disease. J. Clin. Invest. 30: 762–777, 1951.

(25) Shacter, B., Entenman, C., and Shimkin, M. B.: Blood plasma sulfhydryl levels during growth and regression of the Murphy lymphosarcoma of the rat. J. Nat. Cancer Inst. 13: 647–656, 1952.

(26) Shacter, B., Supplee, H., and Entenman, C.: Effect of X-radiation, radiomimetic substances and surgical trauma on sulfhydryl content of plasma. Am. J. Physiol. 169: 499–507, 1952.

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